# CHAPTER 2

# LITERATURE REVIEW

## 2.1 PEPTIDE AND DNA DELIVERY

### 2.1.1 INTRODUCTION

Drug delivery can be of importance for both new chemical entities as well as established drugs. Consequently, drug delivery systems can have a wide variety of applications and benefits. At one extreme it is possible to imagine how a drug delivery system could be instrumental in the successful therapeutic use of a new chemical entity; especially in those situations involving drug targeting or improvement of the absorption of a poorly bioavailable compound from the gastrointestinal tract. Clearly the delivery system will depend on the nature of the material to be delivered (e.g. whole virus, surface protein, and peptide, etc. for a vaccine) and the route (destination), e.g. parenteral, mucosal for the case of vaccines; the muscle, lung endothelia, lung epithelia, liver hepatocytes, tumour tissue, gastrointestinal tract, etc. for gene therapy. For the challenging drugs of the future a wide variety of delivery methods will be required. Unfortunately, but not surprisingly, there is no universal delivery system for peptide drugs, vaccines or for DNA. Each case needs to be considered on its own merits. One should evaluate carefully whether an appropriate delivery system exists already (or can be modified to serve the special needs of the drug) or whether it is necessary to develop a delivery strategy from scratch. The delivery of DNA to specific cells is a clear example of the potential that appropriately designed delivery systems could bring to the success of a new mode of therapy. Here, it will be necessary for the gene construct to be delivered first to the required organ, then to the required cell and subsequently to the required structure within the cell (the nucleus). Gene delivery represents an excellent example of the need for specific delivery and a third order level of targeting. Success will demand a detailed knowledge of different sciences, to include molecular and cell biology, pharmaceutical formulation, colloid science, etc. Today, almost all conceivable routes of delivery are being considered for the delivery of challenging molecules such as peptides, DNA and related polar macromolecular pharmacological agents. For instance, in the emerging field of peptide and protein delivery, molecules that are relatively similar in their characteristics (polypeptides), may present very different opportunities in delivery system development.

This project is focused on delivery systems for challenging drug molecules, which include peptides and DNA. Challenging molecules can also have the opposite physicochemical characteristics, namely compounds that are poorly soluble in water and in a whole range of organic solvents. These compounds are often likened to brick dust' and need to be solubilised. Materials such as cyclodextrins, emulsions, liposomes or the production of small particulates (through methods such as supercritical fluid technology or novel methods of comminution using sophisticated milling and grinding processes) are under active investigation. Many of the new anti-cancer agents, (e.g. taxol), antifungal agents (e.g. itraconazole), are known to be problematical in this regard. In the liposome field, particles coated with polyethylene glycol coating are known to avoid capture by the elements of the reticuloendothelial system and to reach tumour sites and sites of inflammation (probably through processes of passive diffusion rather than active targeting).

### 2.1.2 FUTURE RESEARCH IN DRUG DELIVERY

1. Development of non-viral gene vectors for the delivery of vaccines and therapeutic agents.
2. Surface modified nanoparticles and liposomes for the site specific delivery of anticancer agents, DNA, etc.
3. DNA polymer complexes for drug delivery.
4. Oral delivery systems for targeting to the terminal ileum, ascending colon.
5. Bioadhesive systems that provide retention of drugs in body cavities.
6. Gastroretentive formulations that deliver drugs for local treatment or for enhanced uptake from absorption windows in the small intestine.
7. Nanoparticulate systems for DNA delivery based upon cationic polymers and cationic lipids.
8. Novel permeation enhancers that provide enhanced transport across cells by paracellular and transcellular routes.
9. Oral delivery systems designed to avoid food effects.
10. Particulate systems for approved delivery to mucusassociated lymphoid tissue.

11. Particulate and polymer systems for targeting to tumours following intravenous administration.
12. Targeting systems for lymph nodes.
13. Electrotransport systems for improved transdermal delivery.
14. Liquid and powder injector systems.
15. Polymer implants for conventional drugs and polypeptides.
16. Improved systems for the nasal delivery of drugs used for the treatment of migraine.

## 2.2 LIPOSOMES AS DELIVERY SYSTEMS FOR PEPTIDES AND DNA

### 2.2.1 INTRODUCTION

During the last 26 years since their initial description, (Bangham et al., 1965) liposomes have served a dual role: as a valuable experimental tool for membrane research, (Bangham et al., 1974; Papahadjopoulos et al., 1974; Jost et al., 1982) and in addition, as an *in vivo* delivery system for enhancing the efficacy of various biologically active molecules (Papahadjopoulos, 1978; Knight, 1981). Soon after the delivery of liposomes their application as models of biological membranes and for drug delivery was envisioned (Papahadjopoulos 1978; Gregoriadis 1988) as described in many recent reviews (Lopez-Berestein et al., 1983; Alving et al., 1978). While numerous studies of liposomes as model biomembranes appeared in the mid 1960's, more thorough approaches to drug delivery applications were taken only in the 1970's. Despite an incomplete understanding of their interactions with cells or fate *in vivo*, several early studies showed a great potential of liposomes for drug delivery, in particular treatment of parasitic diseases (Alving et al., 1978; New et al., 1978). Animal studies have shown that liposomes can decrease the toxicity of several antitumor and antifungal drugs (Rahman et al., 1986; Forssen et al., 1981; Olson et al., 1982; Gabizon et al., 1986). Moreover, liposomes have been shown to be efficient carriers of anti-parasitic drugs for treating intracellular infections such as leishmaniasis (Black et al., 1977; New et al., 1978) of macrophage activators for increasing the tumoricidal activity in models of metastasis and of various antigens and adjuvants for enhancing the immune response and thus serving as artificial vaccines. These studies have led to several promising clinical trials.

This preliminary period was followed by an exploration of studies in a period of enthusiastic naivety'. Although rapid uptake of parenterally administered liposomes by phagocytic cells was realized early (Gregoriadis and Ryman, 1972, Beaumier and Hwang, 1983) many researchers underestimated the severity of this limitation, in particular for intravenous medical applications. The uptake is accomplished largely through a scavenger function of cells of the mononuclear phagocytic system (MPS) located in several tissues, originally known as the reticuloendothelial system (RES), which function as an important host defence mechanism. Uptake by these cells usually results in sequestering the drug into the MPS, advantageous for diseases of these tissues or agents which are slowly released back into the blood (Storm et al., 1992), but most often leads to irreversible accumulation and degradation. The loading and leakage characteristics of the desmopressin and insulin containing liposomes, the effect of charge on liposomal entrapment, nasal mucosal permeation of desmopressin loaded liposomes and on pulmonary insulin absorption were investigated by Liu et al., 1993; Hwang et al., 2000; Law et al., 2001.

### 2.2.2 STRUCTURE AND CLASSIFICATION OF LIPOSOMES

Liposomes are simply vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media, giving rise to a population of vesicles, which may range in size from tens of nanometers to tens of microns in diameter. They can be constructed so that they entrap quantities of materials both within their aqueous compartment and within the membrane.

Liposomes can be classified either on the basis of their method of preparation or on the basis of their structural properties. These two classifications are, in principle, independent of each other. Some of the important members of each class are presented in table 2.1.

### 2.2.3 MATERIALS USED IN THE PREPARATION OF LIPOSOMES

#### 2.2.3.1 Phospholipids

Liposomes can be prepared from a variety of lipids and lipid mixtures. Phospholipids (Barenholtz and Crommelin, 1994; Crommelin and Schreir,

1994) are most often used and are the major structural components of the liposomal membranes. Two sorts of phospholipids exist- phosphodiglycerides and sphingolipids, together with their corresponding hydrolysis products. The most common phospholipids are phosphatidyl choline molecules (PC) –amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar head group, phosphocholine. Molecules of PC are not soluble in water and in aqueous medium they align themselves closely in planar bilayer sheets in order to minimize the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Phosphatidyl cholines, also known as lecithin, can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean but less readily extracted from bovine heart and spinal cord. Five groups of phospholipids that can be used for liposome preparation have been described. These include phospholipids from natural sources (e.g. Egg phosphatidyl choline, egg phophatidylethanolamine), modified natural phospholipids (e.g. partially or fully hydrogenated phosphatidyl choline), semisynthetic phospholipids (e.g. phospholipids obtained from natural sources whose acyl chains are removed and chemically replaced by defined acyl chains), fully synthetic phospholipids (e.g. phospholipids made by complete chemical pathways) and phospholipids with non-natural head groups (e.g. polyethylene glycol - phosphatidylethanolamine).

### 2.2.3.2 Sphingolipids

Important members of this group includes sphingomyelins and gangliosides (Lasic et al., 1998)

### 2.2.3.3 Cholesterol

Sterols are important components of most natural membranes, and incorporation of sterols into liposome bilayers can bring about major changes in the properties of these membranes. Cholesterol does not by itself form bilayer structures, but it can be incorporated into phospholipid membranes in very high concentrations –upto 1:1 or even 2:1 molar ratios of cholesterol to PC. In natural membranes, the molar ratio varies from 0.1-1, depending upon the anatomical and cellular location. Being an

amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. To achieve complete control of the properties of the vesicles, particularly high stability and low permeability to the encapsulated substances, it often seems vital to introduce cholesterol and charged lipids, even if the main lipid is inherently effective. The effect of cholesterol on the lateral cohesion of hydrocarbon chains (condensing effect with lipids in the liquid state, fluidizing effect with lipids in the solid state) helps to produce stability of the vesicles over a wide temperature range, as well as low permeability to the encapsulated substances.

#### 2.2.3.4 Charged phospholipids

Negatively charged lipids such as dicetyl phosphate, cholesteryl phosphate, cholesteryl sulfate, phosphatidic acid, phosphatidyl glycerol, gangliomonosides and positively charged lipids such as Stearyl amine, DODAB/C (dioctadecyl dimethyl ammonium bromide / chloride), DOTAP (dioleoyl trimethyl ammonium propane), DOTMA (dioleoyl propyl trimethylammonium chloride), analogues of DOTMA & cationic derivatives of cholesterol (Lasic et al, 1998) etc., improve the stability of the vesicles by preventing their flocculation, and hence their fusion, even in the presence of electrolytes, and help to increase the encapsulation rate of hydrosoluble substances by increasing the thickness of the aqueous layers between the lipid bilayers. As an example, the addition of small proportions of dicetylphosphate to an equimolar mixture of polyglycerol hexadecylether and cholesterol causes an increase in the zeta potential of vesicles dispersed in saline medium and in the main spacing of the lamellar space.

#### 2.2.3.5 Other substances

α-tocopherol and other such antioxidants are often included as components of liposomal membranes to minimize lipid degradation by oxidation (New, 1990a). A chelating agent is also included to sequester heavy metals which act as catalysts for the oxidation reaction (New, 1990a).

### 2.2.4 MECHANISM OF LIPOSOME FORMATION

Liposomes are vesicular structures consisting of hydrated bilayers. The exact mechanisms involved in liposome formation are still not fully understood. It has been suggested that the large free energy change between water and a hydrophobic environment is responsible for the preference of typical lipids to assemble in bilayer structures excluding water as much as possible from the hydrophobic core in order to achieve the lowest free-energy level and hence the highest stability for the aggregate structure (thermodynamic basis of bilayer assembly or the hydrophobic effect) (Lasic et. al., 1998). A high degree of surface activity of a given molecule does not guarantee its ability to form bilayer structures in the presence of water. An attempt was made to identify potential bilayer compounds on the basis of molecular shape by Israelachvili and co-workers (Israelachvili et. al., 1980) but it was a simplification. Yet, this approach to look at packing constrains still helps to predict the behaviour of a new lipid molecule in water. More recent statistical thermodynamic approaches to predict the state of aggregation of phospholipids have been discussed (Seddon, 1990). While such models can give a useful explanation of various liposome morphologies, they obviously had to be expanded. The reasoning of an open bilayered fragment as a transition structure in the vesiculation process was used to explain liposome formation and preparation techniques of liposomes. It is likely that all high-energy treatments of large multilamellar vesicles cause bilayer fragmentation and fragments, after fusion, self-close into unilamellar vesicles (Lasic et. al., 1998).

An alternative "budding off" model used to explain the formation of large multilamellar liposomes from the swelling of dried lipid bilayer. The difference between the area of polar heads on the outer and the inner side of bilayers induce curvature, causes area mismatches and results in budding off when excess of the outer monolayer surface area over the inner one exceeds some critical value. The difference in surface areas can be induced by various gradients such as pH, other ions and the asymmetric presence of molecules that can adsorb or insert into the membrane (Lasic et. al., 1998).

**TABLE: 2.1 CLASSIFICATION OF LIPOSOMES**

**a) Based On Structural Parameters (Size And Number Of Bilayers)**

| Type | Abbreviation | Size range |
|---|---|---|
| Multi lamellar vesicles | MLV | 0.1 –1 μm |
| Oligo lamellar vesicles | OLV | 0.1 –1 μm |
| Small unilamellar vesicles | SUV | 20-100 nm |
| Large unilamellar vesicles | LUV | > 100 nm |
| Giant unilamellar vesicles | GUV | > 1 μm |
| Multivesicular vesicles | MVV | Usually large > 1μm |

**b) Based On Method Of Liposome Preparation**

| Abbreviation | Type |
|---|---|
| REV | Single or oligo lamellar vesicles made by reverse phase evaporation method |
| MLV-REV | Multi lamellar vesicles made by the reverse phase evaporation method |
| SPLV | Stable plurilamellar vesicles |
| FATMLV | Frozen and thawed MLV |
| VET | Vesicles prepared by extrusion methods |
| FUV | Vesicles prepared by fusion |
| DRV | Dehydration-rehydration vesicles |
| BSV | Bubblesomes |
| LUVs-EI | Large unilamellar vesicles prepared by ether injection |
| SUVs-EI | Small unilamellar vesicles prepared by ethanol injection |
| MVL | Multivesicular liposomes prepared by Double emulsion vesicles |

The consensus today is that liposomes are produced whether by self-closure of small bilayered fragments or by fission due to a surface difference between the two opposing monolayers. Some energy input is normally required to form liposomes, which can be described as a kinetic trap that is advantageous as compared to systems at thermodynamic equilibrium.

### 2.2.5 PREPARATION OF LIPOSOMES

A number of methods are available for the preparation of liposomes. Crommelin and Schreir (1994) have given an excellent brief outline of the major methods along with the type of liposome product formed which are listed below:

#### 2.2.5.1 Mechanical dispersion methods

In this group of methods, essentially the simplest in concept, the lipids are dried down onto a solid support (usually the side of the glass container vessel) and then dispersed by addition of the aqueous medium, followed by shaking. (Barenholtz and Crommelin, 1994; Crommelin and Schreir, 1994)

**1. Hand-shaken multilamellar vesicles (MLVs)**

The simplest and the most widely used method of mechanical dispersion is commonly known as hand shaking, since the lipids are dried down on a round bottomed flask and hydrated with the aqueous medium by gentle manual agitation to yield multilamellar vesicles.

**2. Pro-liposomes**

In order to increase the surface of dry lipid while keeping the aqueous volume down, a method has been devised in which the lipids are dried down on to a finely divided particulate support, such as powdered sodium chloride, or sorbitol or other polysaccharide (Payne et al., 1986). Upon adding water to the dried lipid-coated powder (known as pro-liposomes), with mixing on a whirlmixer, the lipids swell, while the support rapidly dissolves to give a suspension of MLVs in aqueous solution.

**3. Freeze-Drying (MLV)**

An alternate method of dispersing the lipid in a finely divided form, prior to addition of aqueous media, is to freeze-dry the lipid dissolved in a suitable organic solvent. The choice of solvent used is determined by its freezing point, which needs to be above the temperature of the condenser of the

freeze-drying. Tertiary butanol is considered as the best suitable solvent. After obtaining the lipid in dry form, in an expanded foam-like structure, water or saline can be added with rapid mixing above the phase transition temperature to give MLVs.

**4. Micro-emulsification liposomes (MEL)**

Recently, the use of a 'microfluidiser' to prepare small MLVs from concentrated lipid suspensions. The fluid collected can be recycled through the pump and interaction chamber until the vesicles of the required dimensions are obtained. After a single pass, the size of vesicles is reduced to between 0.1μm to 0.2μm in diameter, the exact size distribution depending on the nature of components of the membrane and of the hydration medium.

**5. Sonicated vesicles (SUVs)**

To reduce hydrated lipid to vesicles of the smallest size possible, a method generally adopted by exposure to ultrasonic irradiation, which imparts energy at a high level to the lipid suspension. There are two methods of sonication, using either probe or a bath ultrasonic disintegrator. The probe is employed for suspensions, which require high energy in small volume (e.g. high concentrations of lipids, or a viscous aqueous phase) while the bath is more suitable for large volumes of dilute lipids.

**6. French pressure cell liposomes**

One of the first and still very useful techniques developed is the extrusion of preformed large liposomes in a French press under high pressure. This technique yields rather homogeneous uni- or oligo-lamellar liposome preparations of intermediate sizes (30-80nm in diameter depending on the pressure used).

**7. Membrane extrusion liposomes**

A method of reducing the size of liposomes is to pass them through a membrane filter of defined pore size.

**8. Dried-reconstituted vesicles (DRVs)**

Freeze drying method has again been used, but this time, instead of drying the lipids down from an organic solution, a suspension of empty SUVs is

frozen and lyophilized by the addition of cryoprotectants, which on addition of water, can rehydrate, fuse, and reseal to form vesicles with high capture efficiency. This is partly because the water has very ready access to the lipid in this form, and only a small volume need be added to suspend a large quantity of lipid very rapidly.

**9. Freeze-thaw sonication (FTS) method**

A freezing and thawing process is used to rupture and refuse SUVs, during which time the solute equilibrates between the inside and outside, and the liposomes themselves fuse and increase markedly in size, so that their entrapment volume can rise to 30% of the total volume of the suspension (10ul/mg phospholipid).

**10. pH induced vesiculation**

It is an electrostatic phenomenon and the transient change in pH brings about an increase in the surface charge density of the lipid bilayer leads to spontaneous vesiculation.

**11. Calcium induced fusion to produce LUVs**

In this method small vesicles composed of acid phospholipid aggregate in the presence of calcium and subsequently fuse. The flocculant precipitate obtained forms as a result of aggregation of the negatively charged vesicles by the calcium cations. After incubation, the membranes fuse to give extended sheets of phopholipid lamellae.

**2.2.5.2 Solvent dispersion methods**

In this group of methods, the lipids comprising the liposome membrane are first dissolved in an organic solution, which is then brought into contact with the aqueous containing materials to be entrapped within the liposome. At the interface between the organic and aqueous media, the phospholipids align themselves into a monolayer, which forms the basis for half of the bilayer of the liposomes. Methods employing solvent dispersion fall into one of three categories:

**1. Use of water miscible solvents such as ethanol injection (MLV, OLV, SUV)**

An ethanol solution of lipids is injected rapidly into an excess of saline or other aqueous medium, through a fine needle. The force of the injection is usually sufficient to achieve complete mixing, so that the ethanol is diluted almost instantaneously in water, and phospholipid molecules are dispersed evenly throughout the medium. As the solvent concentration is reduced by diafiltration or ultrafiltration, liposomes form. Water miscible solvents like ethanol, glycerine and polyglycols have been employed in preparation of liposomes.

**2. Use of water immiscible solvents such as ether and petroleum ether infusion (solvent vaporisation) (MLV, OLV, LUV)**

The lipid mixture was injected into an aqueous solution of the material to be encapsulated at 55-65°C or reduced pressure. Vaporization of the solvents leads to the formation of single layer vesicles (Deamer and Bangham, 1976).

**3. Water in organic phase**

**a. Reverse phase evaporation**

Liposomes made by this method, developed by Szoka and Papahadjopoulos (1978), and the first to use 'water-in-oil' emulsions, were so called because the process involved an emulsion which was the reverse of the standard 'oil-in-water' emulsion, and because the key, novel step in the preparation was the removal of solvent from the emulsion by evaporation under vacuum. Removal of the organic phase under vacuum causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent under high vacuum or mechanical disruption (vortexing) results in collapse of gel into a smooth suspension of LUVs.

**b. Multivesicular liposomes or Depofoam particle**

A unique feature of the MVV is that inside each depofoam particle, discontinous internal aqueous chambers, bounded by a continuous, non-concentric network of lipid membranes render a higher aqueous volume-to-lipid ratio and much larger particle diameters compared with MLV. MVVs was prepared by evaporation of organic solvents from chloroform-ether spherules suspended in water (Kim et al., 1983)

**2.2.5.3 Methods based on detergent removal by**

1. Gel exclusion chromatography (SUV)
2. Slow dialysis (LUV)
3. Fast dilution (LUV, OLV)
4. Miscellaneous related techniques (MLV, OLV, LUV, SUV)

**2.2.5.4 Methods based on size transformation and fusion**

1. Spontaneous fusion of SUV in the gel phase (LUV)
2. Freeze thawing (MLV)
3. Freeze-drying (MLV)
4. Dehydration of SUV followed by the rehydration with or without sizing
5. Calcium induced growth (LUV, OLV, MLV)
6. Detergent induced growth (LUV, OLV)

### 2.2.6 PROCEDURES FOR THE REMOVAL OF UNENCAPSULATED DRUG FROM THE LIPOSOMAL SUSPENSION

Using liposomes as a pharmaceutical accepted dosage form requires in many cases the complete removal of molecules nonencapsulated in the liposomes. In some cases the removal of free drug is crucial in order to minimize toxicity. In general, much better encapsulation efficiency occurs for lipophilic and/or amphiphilic molecules than for hydrophilic molecules loaded by passive loading, since lipophilic molecules become part of the membrane, the amphiphilic molecules are either membrane associated.

**1. Column chromatographic seperation**

Centrifugation through molecular sieves was first used for the seperation of liposomes from free material with minimum dilution of the sample. Sephadex (e.g. G-50 or G-100), Sepharose or Bio-Gel columns are regularly used to separate liposome-associated material from non-encapsulated material. Sephadex G-50 is the material most widely used for this type of seperation. The liposomes are not retained by the gel bed and elute in the void volume, the free material elutes in the later fractions (New, 1990a; Crommelin and Schreir, 1994). This technique is frequently used for assessment of the encapsulation efficiency.

**2. Dialysis and Ultrafiltration**

Conventional dialysis membranes can be used with molecular weight cutoff characteristics dependent on the molecular weight of the free compound to be removed from the liposome dispersion. Typically, membranes with a molecular weight cut off between 10 and 100kDa are used.

**3. Ultrcentrifugation**

Ultracentrifugation can be used for removal of the unencapsulated material and for separation of heterogeneous colloidal material, be it liposomes with different sizes and / or subfractions with different densities (New, 1990a; Crommelin and Schreir, 1994). It can be used for concentrating dilute liposome dispersions (Barenholtz et. al., 1977). In situations where one entrapping solute in liposomes at high concentration, or where solutions of high molecular weight compounds are used at concentrations iso-osmolar with physiological saline, the density of the suspending medium can easily approach, or exceed, that of the lipid, so that sedimentation is impossible. This problem may be overcome by diluting the liposomes in a medium of much lower density, so that the liposomes become heavier than the surrounding medium by virtue of their content of undiluted solute trapped inside. Ficoll floatation method was employed for the seperation of DNA and proteins (New, 1990).

**4. Protamine aggregation**

This method may be used for the liposomes of neutral and negatively charged. However the solute entrapped inside does not itself precipitate in the presence of protamine after release from the liposomes. The liposomes tend to aggregate in the presence of protamine, leaving the unentrapped material in the supernatant after centrifugation.

**5. Ion Exchange Reactions**

Storm and coworkers (1985) studied the potential of ion exchange resins to remove nonencapsulated material from dispersions with neutral and negatively charged liposomes.

### 2.2.7 CHARACTERIZATION OF LIPOSOMES

Both physical and chemical characteristics of liposomes influence their behaviour *in vivo* and *in vitro*. Several examples demonstrating the importance of proper selection of liposome structures to obtain optimum and reproducible therapeutic effects have been published (Goren *et. al.*, 1990; Senior, 1987; Storm *et. al.*, 1989). Therefore, it is essential to characterize liposomes properly. The nature of characterization of liposomes can be divided into two broad categories.

#### 2.2.7.1 Physical characterization

Physical characterization of liposomes includes evaluation of the following parameter.

**A. Vesicle size**

The size distribution of liposomes is potentially an important characteristic in light of the significant effects vesicle size has on the blood clearance rate. The size distribution of liposomes is often difficult to estimate because often no one technique can monitor all sizes present. More unimodal vesicle size distributions can be achieved by extrusion of the dispersions through polycarbonate filters with a defined pore size. Electron microscopy includes negative staining electron microscopy (New, 1990b), bare grid electron microscopy (Crommelin and Schreir, 1994) and freeze fracture electron microscopy (Betagiri et. al., 1993a). Light scattering techniques (photon correlation spectroscopy), particularly laser-based, quasi-elastic light-scattering methods are becoming popular. These techniques are based on the time-dependent intensity fluctuations of scattered laser light due to the Brownian motion of particles in solution or suspension. For heterogeneous liposome dispersions in the supra-micrometer range Coulter counter techniques can be used. Other techniques that may have advantages for analysing heterogeneous dispersions are based on size exclusion chromatography, high performance gel exclusion chromatography, ultracentrifugation and sedimentation field flow fractionation (Betagiri et. al., 1993a; Crommelin and Schreir, 1994).

**B. Trapped volume**

The trapped volume of liposome preparations (µl/µmol phospholipid) can be found from measurements of the total quantity of solute entrapped inside liposomes and can vary from 0.5µl/µmol for some MLVs and SUVs and 30µl/µmol for certain LUV systems. The best way is to measure the internal volume, therefore, is to measure the quantity of water directly by Karl fisher water content determination of the tight liposomal pellet after centrifugation or this may be done very conveniently by replacing the external medium with a spectroscopically inert fluid (Deuterium oxide- $D_2O$), and then measuring the water signal for example by NMR. The peak height of a NMR is then related to standards consisting of known amounts of $H_2O$ in $D_2O$. Trapped Volumes are usually determined experimentally by dispersing the lipid in an aqueous medium containing nonpermeable radioactive solute such as $^{22}Na$ or $^{14}C$ inulin. The proportion of solute trapped is determined by removing the external radioactivity by centrifugation, dialysis, or gel filtration and determination of the residual radioactivity per lipid (Betagiri et. al., 1993a). Electron spin resonance (ESR) stopped-flow technique is yet another procedure utilised for estimating intravesicular volume (Anzai et. al., 1990).

**C. Lamellarity**

The average number of bilayers present in liposomes can be found by using a number of techniques such as " bare grid" electron microscopy (Crommelin and Schreir, 1994), freeze fracture electron microscopy (Betagiri et. al., 1993a) and negative staining electron microscopy (New, 1990b), $^{31}P$-NMR (Jousma et. al., 1987), trinitrobenzene sulphonic acid method (Barenholtz et. al., 1977; New, 1990b), small angle X-ray scattering (SAXS) (Jousma et. al., 1987) and $^{31}P$ –NMR. Combination of these methods has also been used (Talsma et. al., 1987). In the $^{31}P$ –NMR, the signals are recorded before and after the addition of a nonpermeable broadening agent such as $Mn^{2+}$ ($MnCl_2$). Manganese ions interact with the outer leaflet of the outermost bilayer. Thus the 50% reduction in NMR signal means that the liposome preparation is unilamellar, and 25% reduction in the intensity of the original NMR signal means there are two bilayers in the liposome.

**D. Bilayer fluidity**

The fluidity of the bilayer was also one of the early parameters to be examined in relation to the stability of liposomes *in vivo*. Scherphof and colleagues and others observed that liposomes in blood became unstable by a lipid-exchange mechanism involving lipoproteins). Fortuitously, earlier biophysical work, not connected with drug delivery, had established that cholesterol and the fluidity of lipid bilayers in relation to the main endothermic transition plays an important role in defining the permeability properties of liposomes. The stability of liposomes was further increased by the use of phospholipids with high phase transition temperatures. The bilayer fluidity depends on the bilayer composition, the temperature and the aqueous environment. In particular, in the fluid crystalline state, molecular motion occurs in the bilayer. Information on molecular motion in a bilayer can be collected by florescence polarization techniques. Probes such as diphenylhexatriene (DPH), trimethyl amino-DPH (TMA) or transparinaric acid (NPA) are used. Manipulation of the physico-chemical nature of the probe offers the opportunity to gain information on particular parts of the bilayer structure (Ben-Yashar and Barenholtz, 1989; Jones and Cossins, 1990; Shinitzky and Barenholtz, 1978). Electron Spin Resonance (ESR) Spectroscopy has also been used to determine bilayer fluidity (Crommelin and Schreir, 1994)

**E. Charge**

Surface charge has also been shown to be critical factor in determining the stability and the fate of liposomes *in vivo*. The charge density on liposomes can be estimated from mobility measurements of liposomes in an electrical field (microelectrophoresis). The mobility data can be converted to zeta potentials by using the Helmholtz-Smoluchowski equation or the Henri equation. The zeta potentials can be used to calculate the charge density at the hydrodynamic plane of shear using the Gouy-Chapman equation (Cevc, 1990; Crommelin and Schreir, 1994). Methods that determine that mobility of the individual liposomes in an electrical field visually are now replaced by methods where liposome mobility in an electrical field in a capillary is followed by dynamic light scattering (Grit and Crommelin, 1993).

### 2.2.7.2 Chemical characterization

**1. Analysis of phospholipids and their degradation products**

The quantitative determination of phospholipids can be done by methods like the Barlett assay (New, 1990b), the Stewart assay (Stewart, 1980), the Rhodamine complexation and Thin layer chromatography (New, 1990b). Lysolecithin, a major product of the hydrolysis of lecithin, has been estimated using densiometry. The hydrolysis products of other phospholipids can be estimated in the same way. Estimation of phospholipid oxidation can be obtained using UV absorbance method, thiobarbituric acid method, iodimetric method and gas-liquid chromatography (New, 1990b).

**2. Analysis of cholesterol, α-tocopherol and other bilayer components**

Cholesterol purity can be estimated using gas-liquid chromatography (New, 1990b). Quantitation of cholesterol is done using ferric perchlorate method (New, 1990b) or Zlatkis, Zak & Boyle's method (Zlatkis et. al., 1953). High performance liquid chromatography is used to quantify α-tocopherol (New, 1990b). Different types of chromatography can be used to separate bilayer components such as thin layer chromatography, gas-liquid chromatography, high performance thin layer chromatography and high performance liquid chromatography (Crommelin and Schreir, 1994).

**3. Drug entrapment**

The amount and location of a drug within a liposome depends upon a number of factors such as partition coefficient of the drug between aqueous compartments and lipid bilayers, solubility of the drug in each phase, total amount of lipid used (for nonpolar drugs), internal volume of liposome (for polar drugs) and the method of preparation (Lasic et. al., 1998). The distribution of drug within the liposome may also contribute to level of entrapment and correction of this distribution can increase the entrapment efficacy of the corresponding liposomes (Betagiri et. al., 1993a).

### 2.2.8 STABILITY OF LIPOSOMES

The stability of liposomes can be discussed under the following headings:

### 2.2.8.1 Physical stability

Changes in the size of the liposomes can take place over a period of time. These changes can be a result of aggregation (formation of larger units of liposomal material which is reversible) and sedimentation or fusion (irreversible formation of new colloidal structures). The methods used to characterize the size of liposomes can also be used to follow these changes in size. However, to differentiate between aggregation and fusion, fluorescent markers such as terbium citrate-sodium dipicolinate, calcein-cobalt complex in association with EDTA etc. are used. Liposome membrane fusion may also be detected through fluorescence resonance energy transfer between two lipids originally present in different sets of liposomes (Jones and Cossins, 1990).

Drug molecules can leak from the liposomes. The leakage rate strongly depends on the bilayer composition and the physicochemical nature of the drug. Changes in the bilayer permeability can occur as a result of chemical degradation processes as described below (Crommelin and Schreir, 1994).

### 2.2.8.2 Chemical stability

As phospholipids usually form the backbone of the bilayer, their chemical stability is important. Two types of chemical degradation reactions can affect the performance of phospholipid bilayers:

1. Hydrolysis of the ester bonds
2. Peroxidation of unsaturated acyl chains (if present)

The stability of phospholipids can be ascertained using the methods discussed earlier in the section on characterization of liposomes (Section 2.2.7). The factors that affect the stability of phospholipids (such as pH, ionic strength, temperature and other such factors) will therefore also affect the stability of the liposomes formed from them. Oxidation of cholesterol can be monitored through gas liquid chromatography using a silica capillary column to separate cholesterol from its oxidate products (New, 1990b). The stability of the drug must also be considered since the stability profile of the free" drug may be quite different from its profile in the encapsulated state. There is little information available on the effect of entrapped drug on the stability of phospholipids.

### 2.2.8.3 Approaches to improve liposomal stability

To be commercially feasible, liposomes should have a satisfactory shelf life. In general, a shelf life of atleast one year is a minimum prerequisite in the pharmaceutical industry. Approaches to improve the physical and chemical stability of liposomes are:

**A. Selection of bilayer components**

Stability of liposomes can be improved by minimum use of unsaturated phospholipids. If unsaturated lipids are used, addition of antioxidants such as α- tocopherol or butylated hydroxytoluene and use of argon or nitrogen to minimise exposure to oxygen are recommended. Oxidation of unsaturated phospholipids, catalysed by heavy metals may be inhibited by addition of metal chelators such as EDTA and use of light resistant containers. Processing and storage of liposomes at low temperatures can add to liposome stability. With aqueous liposome dispersions, the selection of a proper pH range (pH 6-7) will reduce hydrolysis and lipid peroxidation mediated damage. Addition of substantial fractions of cholesterol decreases the rate of leakage during storage by rendering the bilayer structure more rigid. Use of saturated phospholipids is a useful technique to improve the stability. To reduce the probability for liposome aggregation or fusion, a charge-inducing agent is often included into the bilayer. Phosphatidyl glycerol (PG), Phosphatidic acid (PA) and charged cholesterol esters such as cholesterol hemisuccinate are negatively charged lipids at neutral pH that have proven to be good candidates for inducing a negative charge on the liposome bilayers. Conversion of small vesicles to larger structures as a result of fusion processes can be markedly reduced by the presence of the trace amounts of the phospholipid isomer 1,3-diacyl-2-phosphatidylcholine. Various methods have been employed which may increase the stability of liposomes by cross linking membrane components covalently using methods such as glutaraldehyde fixation, osmification or polymerisation of alkyne-containing phospholipids. The incorporation of long aliphatic-branched chain polymers (e.g. polyvinyl alcohols esterified with palmitic or stearic acid), up to about 10 % by weight into PC membranes, can substitute for cholesterol in reducing the leakage of medium-sized solutes.

Another type of cross linking, involving hydrogen bonding, is probably responsible for the increased stability of liposomal membranes containing sphingomyelin or 2-carbomoyl PC derivates.

**B. Freeze-drying of liposomes**

Freeze-drying of liposomes is an excellent method to increase the shelf life of liposomes. Cryoprotectants such as sucrose, maltose, trehalose, lactose, proteins, amino acids and polyalcohols have been shown to be effective. Other variables affecting freeze-drying of liposome dispersions include nature of the bilayer and of the liposome-associated compound, liposome size and technological parameters such as freezing rate, freezing temperature and freezing time.

**C. Proliposome approach**

The lipids from an organic solvent are deposited on finely powdered sodium chloride or sorbitol. Upon hydration, liposome dispersion is formed. This approach can only be used for compounds that are fully liposome associated upon hydration and if a rather wide particle size distribution is acceptable in the clinical situation.

### 2.2.9 PHARMACOKINETICS AND FATE OF LIPOSOMES

The first *in vivo* experiments studying the fate of liposome-encapsulated macromolecules were performed 22 years ago and established that intravenously injected liposomes are quickly removed from the blood stream by cells of the mononuclear phagocytic system (MPS), also referred to as the RES, located primarily in the liver and spleen. The liver has the highest rate of clearance followed by the spleen and then the bone marrow. Since then, a lot of work has been done to establish the mechanism of liposomes removal from the circulation, the factors determining the stability of their stability and longevity in blood, and their fate once taken up by various cells. This work has been summarized in reviews by Gregoriadis, Senior, and others. More recently, studies on steric stabilization have yielded liposomes with considerably longer circulation time in blood. Longer circulation enhances liposomes chances for passive extravasation into tumors and for ligand-directed targeting to specific cells. One of the most significant early observations in this field was made by Juliano and Stamp (1975) showing

that small liposomes (SUV) produced by sonication have a much longer circulation time, compared to the larger multilamellar vesicles (MLV). This work indicated that particle size was an important parameter for the fate of liposomes *in vivo.* The RES clearance of large liposomes following IV administration is saturable. Saturation of the uptake process increases liposome circulation time, but does not appear to increase uptake by other tissues. Many drugs have unfavourable pharmacokinetic profiles that may be altered by entrapment in liposomes. It is also possible to alter the pharmacokinetics of the liposomes themselves by varying their size and composition, and/or attaching ligands such as monoclonal antibodies to their surface (Patel, 1988). Liposomes may be engineered to selectively release entrapped drug at target sites through the use of drug release triggered by enzymes, or changes in pH or temperature. The alteration of drug and liposome pharmacokinetics may allow concentration of the drug at the site of action while decreasing the quantity of drug at the site or sites of toxicity (Hwang, 1987). Markers used to determine the pharmacokinetics of liposomes should not be released from the circulating liposomes. Water-soluble markers, such as carboxyfluorescein and certain radioactive markers, are well suited for determining the liposome clearance from the blood. When using radiolabeled markers, a γemitter, such as $^{22}Na$, $^{59}Fe$, $^{111}In$ or $^{99m}Tc$, may be better than βemitters because γemitters avoid quenching problem.

In general, the release of liposome entrapped drug in a tissue is affected by three possible mechanisms.

1. Passive diffusion of drug across the liposome membrane,
2. Perturbation of the liposome membrane by intracellular and extracellular proteins in the tissue,
3. Enzymatic degradation of the liposome membrane.

When using SPECT (Single Photon Emission Computed Tomography) to monitor the degradation of liposome in living mice, the pattern and kinetics of liposome degradation appear to depend upon the route of administration (Hwang and Mauk, 1977; Mauk and Gamble, 1979).

Considering the future prospects of liposomes for *in vivo* applications, it is safe to say that the omens are good, and considering the progress over the last few years, from liposomes that are cleared rapidly by the RES, to long circulating liposomes that can extravasate into tumors and areas of infection, that can be targeted to specific cells within the vasculature and that can show controlled release characteristics following external stimuli such hyperthermia. Future success in most of these areas will require a combination of steric stabilization with either targeting or thermosensitivity, or other external stimuli. Some areas that have not explored as yet *in vivo* include the ability to increase the vascular permeability of specific tissues in a controlled fashion, or to achieve intracellular delivery to target cells following ligand-directed targeting. The former could be achieved by judicious use of inflammatory mediators but the risk of toxicity is still high. The later, i.e. intracellular delivery via liposome-cell fusion is still quite difficult *in vivo* except following the endocytotic events with macrophages and other phagocytic cells. Still, cytoplasmic delivery must involve a fusion event, which was the early aim of some of us in the liposome field. Such fusion although achievable *in vitro* is much more difficult to control *in vivo*. Fusion events are probably related to the transfection effectiveness of DNA-cationic liposome complexes. However, the ability to fuse goes opposite to steric stabiliazation, which inhibits such interactions. The later, nevertheless, is necessary for long circulation *in vivo*, which is crucial for cell-specific targeting. Thus, this is challenge for the next generation of liposomologists to combine steric stabilization with specific for targeting and intracellular delivery *in vivo*.

### 2.2.10 APPLICATIONS OF LIPOSOMES

The literature concerning the applications of liposomes is so vast that a detailed review is not possible. An idea of the varied applications of liposomes can be obtained from table 2.2, which shows the liposomal products developed or under development (Kirby and Gregoriadis, 1999)

**Table 2.2 Liposome-based products developed or under development**

| **Product** | **Drug** | **Target disease** |
|---|---|---|
| Liposomal nystatin | Nystatin | Systemic fungal infections |
| Liposomal tretinoin | All-trans retinoic acid | Leukemia |
| Liposomal annamycin | Annamycin | Kaposis sarcoma<br>Retractory breast cancer |
| Newcastle disease vaccine | Newcastle disease virus (killed) | Newcastle disease (chicken) |
| Avian Rheovirus vaccine | Avian Rheovirus<br>(killed) | For vaccination of breeder chickens; for passive protection of chicks against rheovirus infection |
| AmBisome | Amphotericin B | Systemic fungal infections;<br>Visceral leishmaniasis |
| Amphotec | Amphotericin B | Systemic fungal infections |
| ABELCET | Amphotericin B | Systemic fungal infections |
| DaunoXome | Daunorubicin | First line treatment for advanced Kaposis sarcoma, Breast cancer and other solid tumours |
| Doxil | Doxorubicin | Kaposis sarcoma,<br>Refractory ovarian, recurrent breast, prostate and primary liver cancers |
| D99 | Doxorubicin | Metastatic breast cancer |
| MiKasome | Amikacin | Serious bacterial infections |
| VincaXome | Vincristine | Solid tumors |
| E. coli 1257:H7 vaccine | E. coli 0157:H7 (killed) | E. coli 0157 infection |
| Shigella flexneri 2A vaccine (oral) | S. flexneri 2A (killed) | S. flexneri 2A infection |
| Depocyt | Cytarabine | Lymphomatous meningitis |

| **Product** | **Drug** | **Target disease** |
|---|---|---|
| C53 | Prostaglandin $E_1$ | Systemic inflammatory disease |
| Epaxal-Berna vaccine (IRIV* liposomes | Inactivated hepatitis A virions (HAV) (antigen; RG-SB strain | Hepatitis A |
| Trivalent influenza vaccine | Hemagglutinin and neuraminidase from $H_1N_1$, $H_3N_2$ and B strains according to recommendations by WHO | Influenza |
| HAV/$HBS_s$-IRIV* Combined vaccine | HAV, genetically engineered hepatitis B antigens (HBs) | Hepatitis A and B |
| Diphtheria/ tetanus/ hepatitis A combined vaccine | Diphtheria and α and β tetanus toxoids; inactivated HAV virions | Diphtheria, tetanus, hepatitis A |
| Hepatitis A and B/ diphtheria/ tetanus/ influenza supercombined vaccine (intramuscular) | Inactivated HAV virions, $HB_s$, diphtheria, α and β tetanus toxoids, hemagglutinin and neuraminidase from influenza starins as in trivalent influenza vaccine | Hepatitis A and B, diphtheria, tetanus and influenza |

## 2.3 STERICALLY STABILIZED LIPOSOMES

### 2.3.1 INTRODUCTION

Liposome charge and liposome coating with different polymers, such as PEG, are among the parameters known to strongly affect biological properties of liposomes. The effect of charge and PEG on biological barrier is well investigated (Gabizon and Papahadjopoulos, 1992; Lasic and Martin, 1991). It was repeatedly demonstrated that the incorporation of charged phospholipids into liposomes accelerates their clearance (Lee et al., 1992; Liu and Liu, 1996), while grafting liposomes with PEG and similar polymers make liposomes long circulating (Klibanov et al., 1990; Trochilin and Trubetskoy, 1995). Thus the incorporation of Phosphatidyl serine (PS) or dicetyl phosphate (DCP) into PC/Chol liposomes dramatically enhances liposome uptake by the perfused mouse liver (Liu and Liu, 1996). The fact that the negative charge strongly increases the clearance of liposomes was also shown by Gabizon and Papahadjopoulos (1992). Negatively charged PS was found to abolish the longevity of liposomes prepared of a lipid composition resembling that of the erythrocyte membrane (Allen et al, 1988).

The interest in liposomes has had its advantages and disadvantages. The pharmaceutical and pharmacological justifications of the use of liposomes as drug carriers are as follows (Barenholtz and Crommelin, 1994).

1. Liposomes supply both a lipophilic environment and aqueous *'milieu interne'* in one system and therefore suitable for the delivery of lipophilic, hydrophilic and amphiphathic molecules.
2. Liposomes are biocompatible due to their biodegradability, low toxicity and lack of immunogenicity.
3. Liposomes can be administered through most route of administration including ocular, pulmonary, nasal, oral, intramuscular, subcutaneous, topical and intravenous.
4. Pharmacokinetics and *in vivo* biodistribution of liposomes can be controlled by their port of entry combined with their lipid composition and size.

Till now, one of the main drawbacks to the use of liposomes as a parenteral drug delivery system was their rapid uptake from the circulation by cells of MPS as a result of liposome opsonisation in the blood. Due to this, the therapeutic use of liposomes had been limited mainly to delivery of drugs to the MPS e.g. manipulation of macrophage function and treatment of diseases involving macrophages like leishmaniasis. However, this scenario changed with the advent of what are known as sterically stabilized liposomes as a means to impede opsonisation and MPS uptake. Long circulation is frequently cited as a major advantage of macromolecular injectable drugs. Liposomes serve as a good model for the understanding of grafted polymer influence on carrier properties, and much regularity found for liposomes might be successfully applied to many microparticulate drug carriers. One of the most popular and successful methods to obtain long-circulating biologically stable liposomes is their coating with certain hydrophilic and flexible polymers, first of al with polyethylene glycol (PEG).

### 2.3.2 AGENTS USED FOR STERIC STABILISATION

Many recent reports have demonstrated that rapid uptake of liposomes *in vivo* by cells of the mononuclear phagocytic system (MPS), which has restricted their therapeutic utility, can be overcome by incorporation of lipids derivatised with the hydrophilic polymer polyethylene glycol (PEG). The structure-function relationship of PEG-derivatised phosphatidyl ethanolamine has been examined by measurement of blood lifetime and tissue distribution in mice and rats. PEG possesses an ideal array of properties:

1. Very low toxicity
2. Excellent solubility in aqueous solutions
3. Extremely low immunogenicity and antigenicity
4. Non-biodegradable but readily excretable in living organisms
5. Possess excellent pharmacokinetic and biodistribution behaviour
6. Readily available in a variety of molecular weights
7. Inertness of polyether backbone in biological environments as well as in most chemical reaction conditions under which the end groups of PEG can be subjected to chemical modification and/ or conjugation reactions.

8. When injected into animals it shows high persistence in blood compartment and low accumulation in reticuloendothelial system (RES) organs, liver and spleen. In addition it is remarkable *in vivo* behaviour, the well known propensity of PEG to exclude proteins, other macromolecules, and particulates from its surroundings is one of the principal reasons for use of this polymer for preparation of various conjugates such as PEG coated liposomes for drug delivery. PEG coated liposomes have several advantages.

1. It is easy to prepare and use without modification of the many well-known liposome methodologies.
2. A coating with PEG not only dramatically increased the liposomes blood half life but also solved some problems related to the use of GM1 such as its costliness and its difficulty in purification.
3. Increased stability to contents leakage
4. Increased flexibility in lipid composition
5. Amenable to pharmaceutical product regulatory requirements
6. Compatibility with ligands or other chemical functionalities at the outer surface of the PEG coating.
7. PEG-grafted liposomes not only can keep drugs in the blood but they also can deliver them to several important pathological tissues, including solid tumors, infections, and apparently most sites of active inflammation which can result in greatly enhanced efficacy of encapsulated drugs.

PEG-lipid conjugates have been synthesised and incorporated into liposomes to form a steric polymer surface barrier that enhances drug delivery applications. Upon parenteral administration, liposomes are diluted extensively into body fluids such as blood, lymphatic or extracellular fluid. The exact amount depends on the volume of the original sample, the animal size and the route of administration. The opsonin proteins of the plasma, as well as lipoproteins and phospholipases, are by far the most important factor in alterations with the destabilisation of liposomes. Liposomes may serve as a good model for the understanding of grafted polymer influence on carrier properties, and many regularities found for liposomes might be successfully applied to particulate drug carriers. One of the most popular and successful methods to obtain long-circulating biologically stable

liposomes is their coating with certain hydrophilic and flexible polymers, first of all with poly(ethylene glycol) (Klibanov et al., 1990; Mori et al., 1991; Torchilin et al., 1992). To make PEG capable of incorporation into the liposomal membrane, the reactive derivative of hydrophilic PEG is a single terminus-modified with hydrophobic moiety (usually, the residue of phosphatidyl ethanolamine or long chain fatty acid is attached to PEG-hydroxy-succinimide ester, PEG-OSu) (Klibanov et al., 1990).

Most of the PEG-lipids used for preparation of long circulating liposomes are prepared by derivatization of phosphatidylethanolamines (PEs), which act as an anchor to graft the PEG chain to the liposome surface. Monomethyl ether of PEG (mPEG) is often used for conjugation. It is particularly useful when multiple chains of the polymer have to be linked to the intended substrate. Due to its structural simplicity and possession of only one derivatizable end group, the use of mPEG minimizes crosslinking possibilities and leads to improved homogeneity of conjugates. Figure 2.1 shows various reagents used for derivatization of PE and the end products so obtained (Zalipsky, 1995).

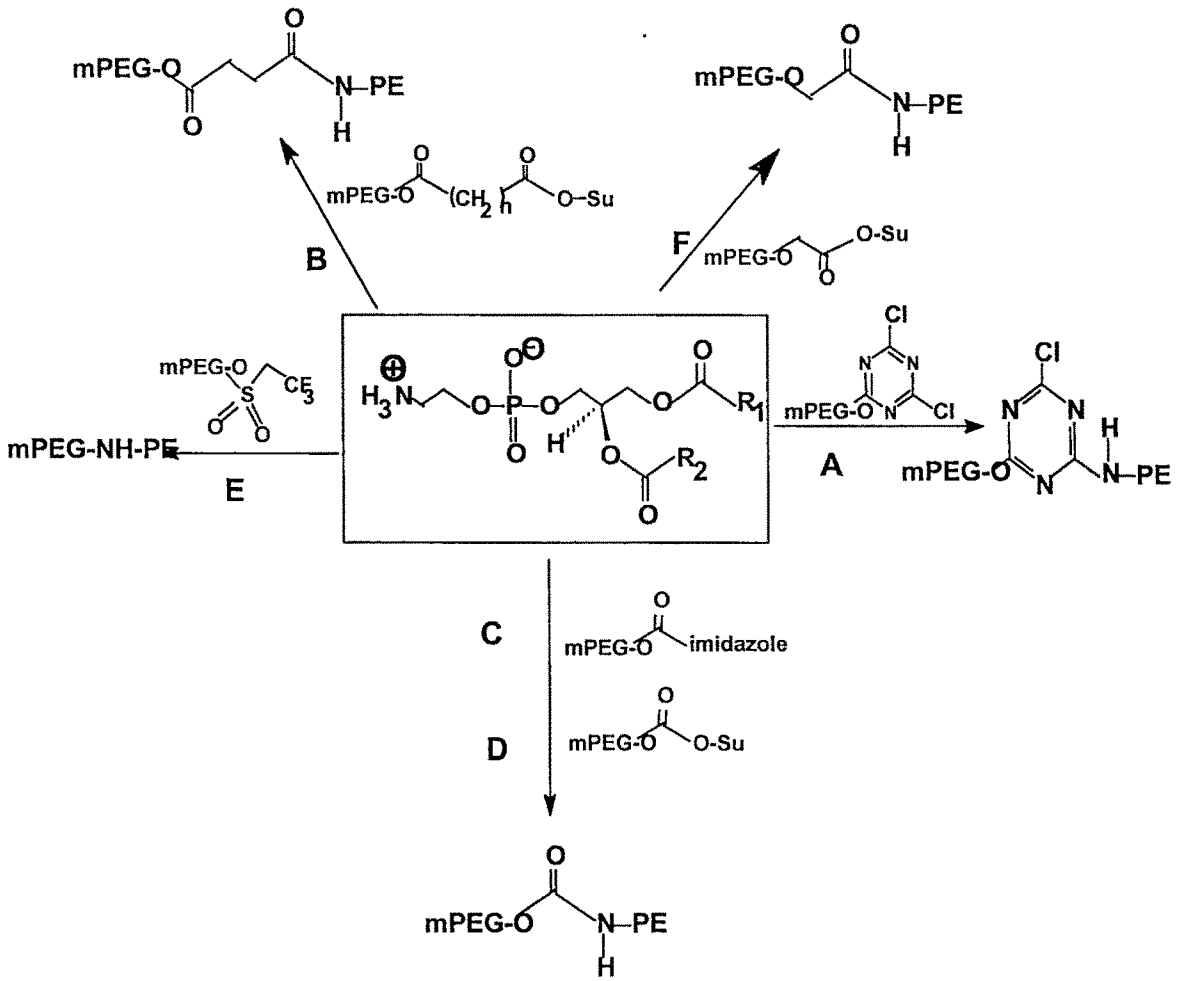
The chlortraizine approach (A, figure 2.1) to the preparation of PEG-PE (Blume and Cevc, 1990) is not attractive due to the toxicity of the reagent and its degradation products. The presence of slightly reactive chloride in the PE conjugate with (A) is another drawback of this method.

Klibanov and coworkers used succinimidylsuccinate-PEG (SS-PEG) (B, figure 2.1) derived from mPEG-5000 for preparation of mPEG-succinamide-dioleoyl-PE (Klibanov, *et. al.,* 1990). Succinate ester linkage present in this conjugate is known to undergo hydrolysis under physiological conditions (Zalipsky, 1995). Parr and coworkers (Parr, *et. al.,* 1994) demonstrated that this type of conjugate readily releases free mPEG-OH after a few hours of incubation at 37°C in mouse serum.

Urethane linked mPEG-PE conjugates were obtained by reacting mPEG-oxycarbonylimidazole (C, figure 2.1) or succinimidyl carbonate (SC-PEG) (D, figure 2.1) with distearoyl-PE (Allen *et. al.,* 1991; Woodle *et. al.,* 1992). The reagent (D, figure 2.1), being more reactive, allows preparation of mPEG-

DSPE within 10-15 minutes at 45°C. Urethane linkages are quite stable and thus are more attractive than other linkages (Zalipsky, 1995).

**Figure 2.1 Methods for the preparation of mPEG- phosphatidyl ethanolamine conjugates**




DSPE (distearoylphosphatidylethanolamine)-PEG conjugates derived from reagent (E) (mPEG-tresylate) of molecular weight 5000 were prepared for formulation of long circulating liposomes (Tilcock *et. al.,* 1993; Blume and Cevc, 1990). Instead of using a PEG-lipid conjugate to form liposomes, Senior and coworkers (Senior *et. al.,* 1991) modified surface amino groups of DSPC (distearoylphosphatidylcholine) ĐPPE (dipalmitoyl-PE)-cholesterol vesicles with this reagent. The attractive feature of this approach is in its

selective grafting of the polymer on the exterior of the vesicles. It avoids the presence of mPEG residues inside the liposomes.

Amide linked mPEG_DSPE was prepared by coupling succinimidylester of carboxymethylated PEG (F, figure 2.1) to the amino group of PE (Parr *et. al.*, 1994).

### 2.3.3 PROPERTIES OF PEG-GRAFTED LIPOSOMES

The biological properties of PEG coated liposomes advantageous for drug delivery is a consequence of physiochemical interactions of the polymer with protein and cells.

#### 2.3.3.1 Physiochemical properties

A steric barrier is thought to be formed on the surface of PEG-grafted liposomes, which inhibits protein adsorption, particle opsonisation, and concomitant MPS uptake. Physical measurements are beginning to substantiate this mechanism but further investigation and refinement are needed. PEG-grafted liposomes also referred to here as sterically stabilized liposomes (SSL). PEG was one of the first synthetic polymers examined to graft onto the surface of liposomes for the purpose of prolonging blood circulation. It is still preferred despite early expectations that other hydrophilic polymers would give similar effects. Attempts to use dextran for similar increase in blood circulation apparently were not successful while some success was reported with polyvinylpyrolidone and polyglycerols (Torchilin et al., 1994; Maruyama et al., 1994). These polymers showed similar effects but none proved to be equal to PEG. More recently, other polymers equal to PEG have been identified: two forms of polyoxazolines, which represent a very different class of water soluble polymers (Woodle et al., 1994; Zalipsky et al., 1996).

PEG-PE can form micelles giving clear solutions in water even upto concentrations as high as several mg/ml. Thus, PEG-PE has the potential to dissociate from the liposome and form micelles in equilibrium with bilayers. This problem' has been taken advantage of to achieve a slow loss of PEG-coating by selection of the lipid anchor. A reversal of this process, insertion of PEG-PE from micelles into preformed liposomes, also has been used as a means to prepare liposomes with a PEG coating (Woodle, 1997).

Introduction of PEG-PE replacing the distal methoxy with chemical functionalities useful for conjugation is an important advancement. The need for such functionalised PEG lipids, shown in figure 2.2, was created by their usefulness in attachment of various biological relevant ligands to the exterior of the PEG-grafted liposomes. Some of the end group functionalised PEG-lipids have already been successfully used in various conjugation protocols resulting in attachment of immunoglobulins, peptides and other ligands to the distal ends of liposome-grafted PEG chains (Zalipsky, 1995).

Carboxy-PEG-DSPE was prepared by coupling bis-carboxyl-PEG to the amino group of PE followed by isolation of the desired product (I, figure 2.2) by silica gel chromatography. It has been used for conjugation of plasmogen to liposomes for targeting to fibrin. Longevity in plasma circulation was well preserved (Blume and Cevc, 1993; Blume *et. al.,* 1993). This was the principle methodology for preparing some of the other conjugates (II-IV) (figure 2.2).

Amino-PEG-DSPE (II, figure 2.2) has proved useful as starting material for synthesis of other functionlilzed PEG-lipids. E.g. 2-pyridylthiopropionamide (IV, figure 2.2) and bromoacetamide (V, figure 2.2) derivatives. Amino-PEG-DSPE was cleanly derivatized with heterobifunctional reagents N-succinimidyl pyridyl dithiopropionate) (SPDP) and p-nitrophenyl bromoacetate respectively. Placing an amino acid residue as part of Linker A (figure 2.2) is helpful for the final chemical characterization of the conjugates by amino acid analysis. This was utilized in case of hydrazide-PEG-DSPE (III, figure 2.2). Amino-PEG-DSPE, apart from being used for preparing other end group fuctionalized PEG-DSPE, also forms long circulating liposomes, behaving as positively charged particles (Zalipsky *et. al.,* 1994).

Hydrazide-PEG-DSPE (III, figure 2.2) form hydrazone linkages with aldehydes. It is used for conjugation of antibodies, oxidized on their carbohydrate residues, to distal ends of PEG chains on surfaces of liposomes. It is also used for conjugation of periodate treated N-terminal Serine or Theorine peptides (Zalipsky *et. al.,* 1996).

2-pyridyldithiopropionamide-PEG-DSPE (IV, figure 2.2) has been used as a prescursor for HS-PEG-DSPE. It is very efficient for attachment of maleimide-containing antibodies to end groups of PEG on liposomal surfaces. It is used for binding HS-containing ligands through disulfide linkage (Allen *et. al.,* 1995a).

Bromoacetamide-PEG-DSPE (V, figure 2.2) is an efficient moiety for thioether forming reactions with HS-ligands. It is utilized for Fab-SH attachment to end groups of PEG chains on liposomes (Zalipsky, 1995).

Linker B in most of the above cases (figure 2.2) was a stable urethane linker formed by reacting PE with an appropriate succinimidyl carbonate-PEG-derivative (SC-PEG-X) where X represents a functional residue or a protected form thereof. This might allow further control of the *in vivo* properties of the relevant conjugated liposomes (Zalipsky 1995).

Certain new polymers have also been suggested for the steric protection of liposomes. The list is presented as figure 2.3 (Torchilin and Trubetskoy, 1995). A number of methods have been used to conjugate PEG to lipids, described in recent reviews (Zalipsky, 1995). A common approach has been to react activated PEG with the primary amine of PE. Mono-functional methoxy-PEG reactive at only one end reduces the number of potential products. PEG-PE containing a carbamate or urethane linkage has good stability characteristics (Parr et al., 1994) and is commercially available. Note that chemical differences can exist between molecules all referred to as PEG-PE yet the overall nature of these molecules is quite similar. For example, several methods can be used to conjugate PEG to PE but most eliminate protonation of the amine in aqueous solution. Thus these result in different chemical species but with similar pharmacokinetic properties.

**Figure 2.2 End group functionalized PEG lipids**

**X -Linker A -PEG -Linker B -DSPE**

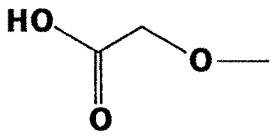
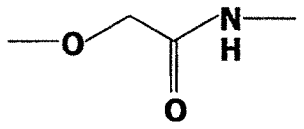
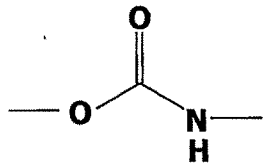
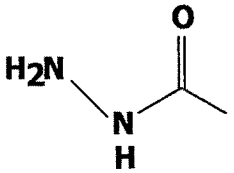
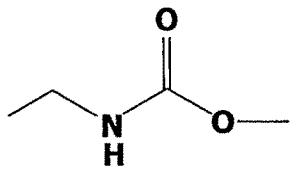
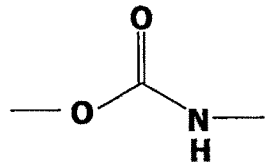
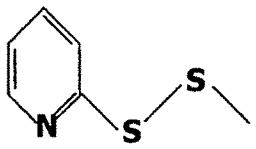
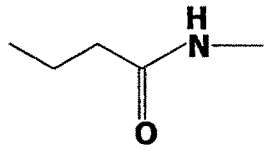
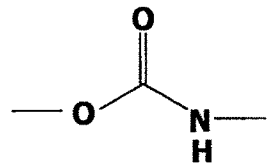
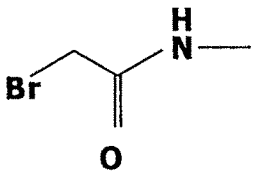
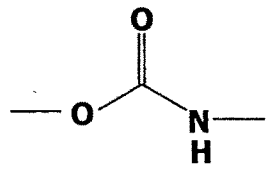
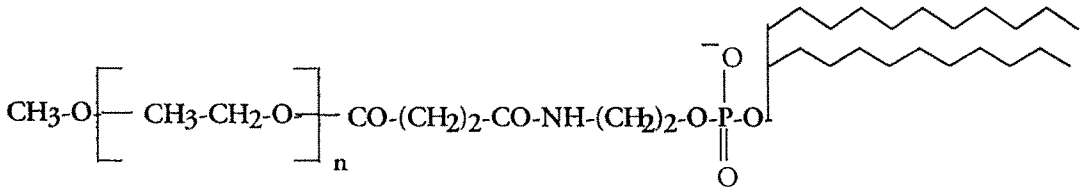
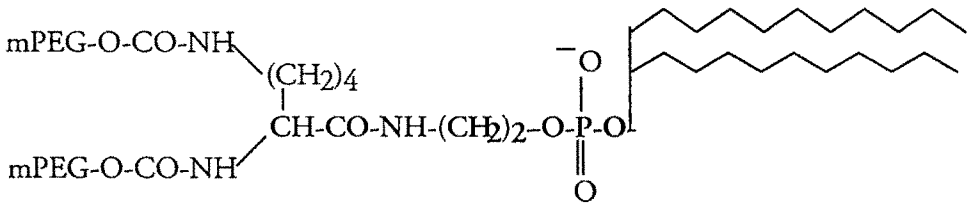
| No. | X- | -Linker A - | -Linker B - |
|---|---|---|---|
| I |  | None |  |
| II |  | None |  |
| III |  |  |  |
| IV |  |  |  |
| V |  | None |  |

**Figure 2.3 Amphiphilic synthetic polymers used for steric protection of liposomes**



PEG-PE



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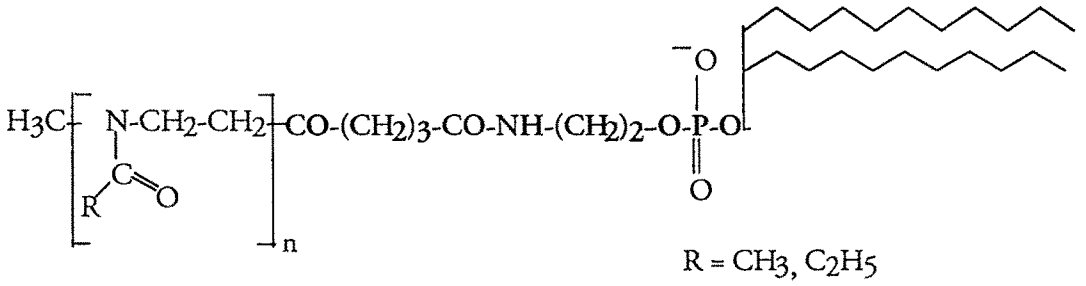
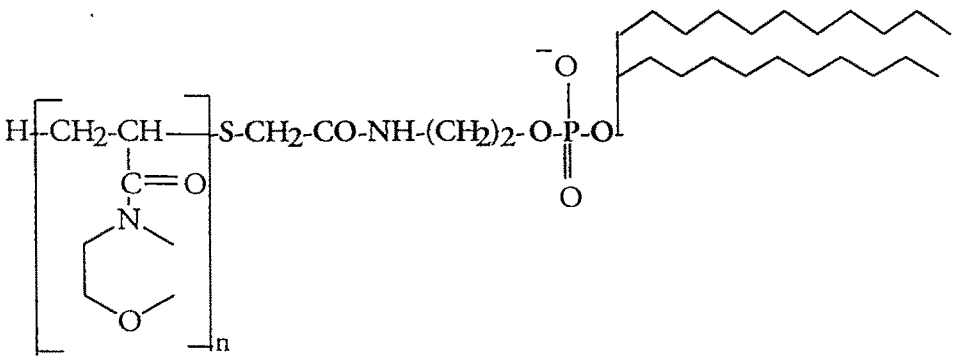


Poly(2-alkyl-2-oxazoline-)-PE



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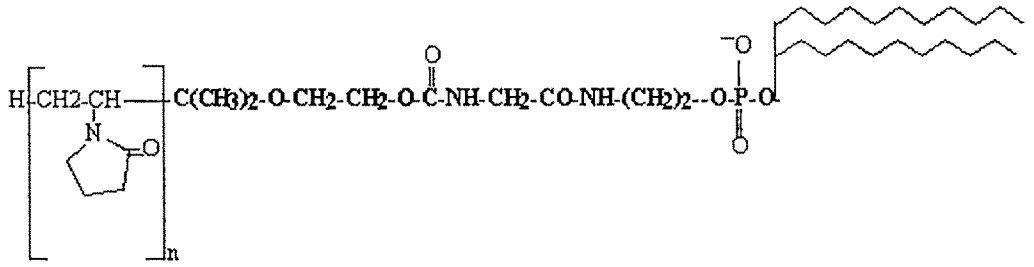
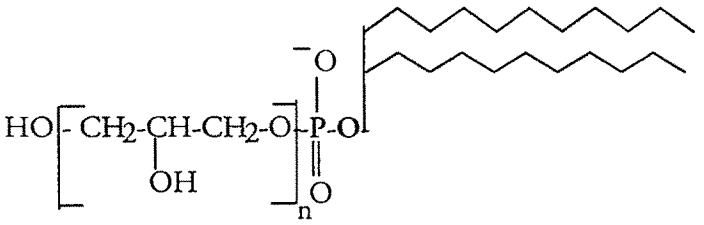
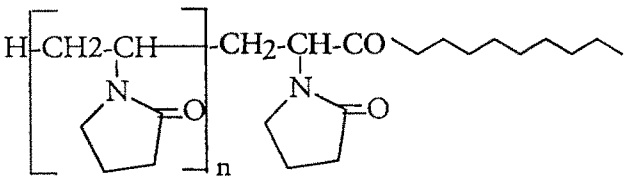


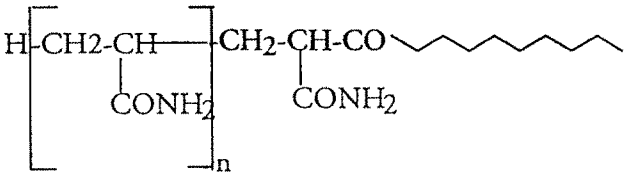
Poly (vinyl pyrrolidone)-PE



Poly (glycerol)-phosphatidyl glycerol



Poly (vinyl pyrrolidone)-palmitate



Poly (acryl amide)-palmitate

### 2.3.4 THE IMMUNE SYSTEM

To understand the behaviour of sterically stabilized liposomes, a brief overview of the human immune system is necessary. The human body has the ability to resist almost all types of organisms or toxins that tend to damage the tissues and organs, which is called immunity. While a portion of the immunity results from general processes and is known as innate immunity, the major portion of the immunity results from a special immune system, which produces antibodies and activated lymphocytes that attack and destroy specific organisms or toxins. This latter type of immunity is known as acquired immunity.

#### 2.3.4.1 Innate immunity

Innate immunity includes the following:

Phagocytosis of bacteria and other invaders by white blood cells and cells of the tissue macrophage system. The white blood cells or leukocytes are the mobile units of the body's protective system. The various types of leukocytes include polymorphonuclear neutrophils, polymorphonuclear eosinophils, polymorphonuclear basophils (collectively these three types are known as granulocytes), monocytes, lymphocytes and plasma cells. In addition, there are a large number of platelets, which are fragments of a seventh type of white cell found in bone marrow, the megakaryocyte.

It is mainly the neutrophiles and monocytes that attack and destroy injurious agents. The neutrophils are mature cells that can attack and destroy such agents in the circulating blood. However, the blood monocytes are immature cells that have very little ability to fight with injurious agents. However, once they enter tissues, they swell too much larger sizes to become macrophages. While mainly these cells are mobile, a large portion of these macrophages become attached to the tissues and remain attached for months or perhaps even years unless they are called upon to perform specific protective functions. When appropriately stimulated, these fixed macrophages can break away from their attachments and become mobile macrophages. The combination of mobile macrophages and fixed tissue macrophages is collectively called the reticuloendothelial system or the RES. The tissue macrophages in various tissues differ in appearances and they

are known by different names: Kupffer cells in the liver; tissue macrophages in the lymph nodes, spleen and bone marrow; alveolar macrophages in the alveoli of the lungs; tissue histocytes in the subcutaneous tissues and microglia in the brain. The term mononuclear phagocytic system (MPS) has been proposed to replace RES.

**2.3.4.2 Acquired immunity**

The human body also has the ability to develop extremely powerful specific immunity against individual agents such as lethal bacteria, viruses, toxins and even foreign tissues from other animals. This is called acquired immunity. Two basic, but closely allied, types of acquired immunity occur in the body:

1. Humoral immunity: Here, the body develops circulating antibodies, which are globulin molecules that are capable of attacking the invading agent.

2. Cell-mediated immunity: Here, large numbers of activated lymphocytes are formed that is specifically designed to destroy the foreign agents.

Both the antibodies and the activated lymphocytes are formed in the lymphoid tissue of the body, which is located most expensively in the lymph nodes, spleen, submucosal areas of the gastrointestinal tract and in the bone marrow. The lymphoid tissue is distributed very advantageously in the body to intercept the invading organisms or toxins before they can spread too widely. The lymphocytes if normal lymphoid tissues are divided into two major populations:

A. T-lymphocytes: These forms activated lymphocytes and thus are responsible foe cell-mediated immunity.

B. B-Lymphocytes: These form antibodies and thus are responsible for humoral immunity.

**A. T-cell mediated immunity**

Upon exposure to the proper antigen, the T-lymphocytes of the lymphoid tissue proliferate and release large numbers of activated T-cells into the lymph. These then pass into the circulation and are distributed throughout

the body passing through the capillary walls into the tissue spaces, back into the lymph and blood, thus circulating throughout the body.

T-cells are of different types such as:

**1. Cytotoxic T cells**

These are direct attack cells capable of killing micro organisns and at times even the body's own cells. For this reason, they are frequently called killer cells.

**2. The Helper T cells**

These cells are the most numerous of all the T cells. They help is functions of the immune system in various ways.

i. By increasing the activation of B cells, cytotoxic T cells and suppressor T cells by antigens. This is done by secretion of lymphokines by the helper T cells once they are activated.
ii. Stimulation of activity of other T cells by secretion of a substance called interleukin-2, one of the lymphokines, that increases the activity of other T cells, including cytotoxic T cells, some suppressor T cells and probably even some of the other helper T cells.
iii. Activation of the macrophage system by secretion of another lymphokine, called macrophage migration inhibition factor that slow or stops the migration of macrophages that have been chemotactically attracted into the infected tissue area thus causing a great accumulation of macrophages. This factor also activates the macrophages to cause far more efficient phagocytosis allowing them to attack and destroy greatly increased numbers of invading organisms.

**3. Suppressor T cells**

These cells regulate the activities of the other cells keeping them from causing excessive immune reactions that might be severely damaging to the body. They are therefore also called regulatory T cells. These cells also play a role in immune tolerance.

**B. B-cell mediated immunity**

Upon entry of a foreign antigen, the lymphoid tissue macrophages phagocytize the antigen and then present it to the adjacent B cells. In

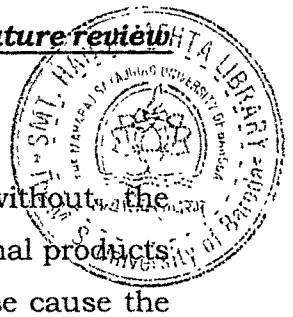
addition, the antigen may also be presented to T cells at the same time, the consequences of which have been discussed earlier. The B-lymphocytes specific for the antigen develop to give plasma cells, which produce antibodies. These antibodies are gamma globulins (also known as immunoglobulins). Some of the progeny of the B-lymphocytes do now form plasma cells but form moderate numbers of new B-lymphocytes similar to the original cell (memory cells). Due to the presence of these cells, subsequent exposure to the same antigen will the cause a much more rapid and much more potent antibody response.

There are five general classes of antibodies namely IgM, IgG, IgA, IgD and IgE. Antibodies act mainly, in two different ways to protect the body against invading agents:

1. By direct attack on the invader: Antibodies can inactivate the invader by agglutination, precipitation, neutralization or lysis.
2. By activation of the complement system that then destroys the invader: Complement is a collective term to describe a system of about 20 different proteins, many of which are enzyme precursors. All these are present normally among the plasma proteins that leak out of the capillaries into tissue spaces. The enzyme precursors are normally inactive but they can be activated through two separate ways:

**a. The Classical pathway**

This pathway is activated by an antigen-antibody reaction. I.e. when an antibody binds with an antigen, a specific reactive site on the antibody is uncovered or activated and this, in turn, binds with a molecule of the complement system setting in motion, a "cascade" of sequential reactions which, starting from a small amount, lead to the production of large amounts of a number of end products. These cause effects such as opsonization and phagocytosis, lysis, agglutination, neutralization of viruses, chemotaxis, activation of mast cells and basophils and inflammatory effects, all of which help to prevent damage by the invading organism or toxin.



**b. The Alternate pathway**

The complement system can sometimes be activated without the intermediation of an antigen-antibody reaction. All the same final products of the system are formed as in the classical pathway and these cause the same effects as those just described above in protecting the body against the invader. Since the alternate pathway does not involve an antigen-antibody reaction, it is one of the first lines of defence against invading agents.

### 2.3.5 OPSONIZATION AND PHAGOCYTOSIS

Opsonization is a process whereby materials adsorb to a foreign surface in such a way as to prepare the surface for recognition as foreign and thus for phagocytosis by the MPS. The word 'opsonin' is of Greek origin and means a relish, a seasoning or a sauce. It has been quoted as being "the preparation of meal"(Buckton, 1995). The materials involved in opsonization are known as opsonins. Opsonins are poorly defined plasma factors, which bind to foreign particles and promote phagocytosis. The opsonins facilitate attachment of the phagocyte to the antigen by forming a bridge between the phagocyte and antigen. These opsonins can be specialized or non-specific. Specialized opsonins contain at least one receptor-recognizable site and often more than one binding site (so that they can crosslink). They also play important roles in immune response regulation and may alter vascular permeability. Boundary interactions *in vivo* are often mediated by components of biological fluids (blood, interstitial liquid), cells and the extracellular matrix. For example, particle clearance from blood is most often attributed to uptake by phagocytes, e.g. in liver and spleen. Phagocytosis may be mediated by specialized recognition proteins, such as immunoglobulins (Atkinson et al., 1974), fibronectins (Walton et al., 1984), complement system (Law et al., 1980), soluble lectins (Ezekowitz et al., 1988), vitronectin (Savill et al., 1990), etc. Several recognition proteins have been shown to be able to bind liposomes, cell membranes or phospholipids. Opsonin-mediated phagocytosis may have an element of cooperativity, because the phagocytosis rate depends on the number of opsonin molecules in the immunocomplex (Ehlenberger et al., 1977). In some cases, opsonins

trigger phagocytosis only in cooperation with other factors present in the immunocomplex (Ross et al., 1985). The distinctive feature of recognition proteins relates to their capability to enhance polymer binding and uptake by phagocytes (Brown et al., 1970). Specialized opsonins, bound to an antigen, expose receptor-recognizable sites and, thus mediate further immunocomplex interaction with cells. The latter process depends on factors such as the number and type(s) of incorporated opsonins molecules. Those immunocomplexes, which contain, a small number of opsonins molecules are known to circulate for hours and even days while others are immediately arrested by phagocytes. (e.g. in liver, spleen, bone marrow or lungs). Thus phagocytosis is triggered faster by multiple rather than single opsonins -receptor interactions (Papisov, 1995).

**2.3.5.1 MAJOR SPECIALIZED OPSONINS OF NORMAL BLOOD INCLUDE**

Major specialized opsonins of normal blood, such as immunoglobulins, proteins of complement system and fibronectins.

**1. Immunoglobulins**

Immunoglobulins are the most specialized opsonins that can recognize and selectively bind specific fragments of macromolecular species. Immunoglobulins have high affinity for antigens known' to the immune system of a certain individual. The major concern regarding liposome pharmacokinetics (and integrity) relates to opsonins specific to phospholipid-protein complexes (Senior et al., 1986).

**2. Complement**

Complement comprises a set of proteins that work to eliminate microorganisms and other antigens from blood. This task is achieved by complement cooperation with cells that express complement has historically assigned numbers, which do not completely correspond to the sequence of their actions. The third complement protein, C3, has a central role in complement function. One of the most important functions of complement is to mark antigens with fragments of C3, thus making them recognizable to phagocytes bearing C3 receptors (Papisov, 1995). To be bound to an antigen, C3 should be first activated by C3 convertases (which are antigen bound) that produce C3b, a C3 fragment containing an active cyclothioester

group; the latter is chemically active and may covalently bind nucleophilic functional groups, preferentially OH groups (Law et. al., 1980). C3 convertases are protein complexes formed either with participation of activated C1 complex, C2 and C4 (C4b2a, classical activation pathway) or by C3 itself and factors B and D (C3bBb, alternative pathway). Subsequent C3 activation produces more antigen-bound C3b and more C3 convertase complex (Papisov, 1995). Other complements such as C4 also seem to be involved in the phagocytic process (Gref et. al., 1995). C4, which is less abundant, has a thioester group, similar to that in C3, which binds, however, preferentially amino groups (Law et. al., 1984). The antigen ability to activate complement presumably depends on the presence of nucleophilic functional groups that may bind C3 or C4 and/or domains that may bind C1. the C1 complex consists of three proteins C1q, C1r and C1s where C1q substances including IgG and IgM – containing immune complexes, lipopolysaccharides and porins from gram-negative bacteria, ligand-bound C-reactive protein, nucleic acids and other highly charged polymers (Papisov, 1995).

**3. Fibronectins**

Fibronectins are large proteins abundant in plasma and extra cellular matrix. These are indications that the fibronectin molecule in the solution is a disk having a diameter of 30 nm and thickness of 2 nm (Benecky et. al., 1990). However, the fibronectin molecule consists of several domains connected by relatively flexible joints and believed to be capable of unfolding, in particular, as a result of substrate binding (Khan et. al., 1990; Wolff and Lai, 1989).

**2.3.5.2 MINOR OPSONINS**

Minor or non-specific opsonins are proteins present in blood in small concentrations. These are recognition proteins bearing binding sites, receptor –recognizable domains and sometimes, sites for binding another recognition molecule. Some minor opsonins are acute-phase proteins (i.e. their concentration in plasma increases as a result of inflammation or trauma). Many minor opsonins trigger complex responses that often include histamine release (Papisov, 1995). Most minor opsonins are lectins. e.g.

conglutinin is a 330 kDa lectin with four binding sites and high specificity to terminal (α1,2) oligomannose (Young and Leon, 1987). Lipopolysaccharide binding protein (LBP) was recently shown to directly recognize lipopolysaccharides and to trigger remarkable biological responses (Tobias et. al., 1993). Vitronectin, a component of extracelluar matrix, participates in complement reactions (often named protein S) and probably may also act as opsonins (Savill et.al., 1990). Serum amyloid P component (SAP) is a normal human serum protein with the non-fibrillar amyloid P component found in amyloid deposits. SAP is composed of 10 non-covalently associated globular subunits, e ach of 25 KD as arranged as two face-to-face cyclic pentameric discs. SAP binds to a number of apparently unrelated substances including phospholipids (Saxena et. al., 1987). Another pentraxin, C-reactive protein (CRP) is an acute phase protein. Human CRP interacts with fibronectin, fibrogen and phosphorycholine; rat CRP binds phosphorylethanolamine as well (Kottgen et. al., 1992). Other minor opsonins are tuftsin, which has been shown to enhance recognition of various particulates by different macrophages (Stolnik et. al., 1995).

Recently, the presence of 'organ-specific' opsonins has been proposed (Moghimi and Patel, 1998) where, for instance, the liver specific opsonins can enhance the uptake of particulates by kupffer cells whereas spleen specific opsonins could mediate the uptake of particulates by spleen macrophages.

It has been suggested that phagocytosis is regulated by the presence and balance between two groups of blood components: opsonins, which promote phagocytosis and dysopsonins that, suppress the process. Immunoglobulin IgA and Secretory IgA are the best-known dysopsonins.

### 2.3.6 AVOIDANCE OF MPS UPTAKE AND ITS CONSEQUENCES

The current understanding of liposome biological interactions is based primarily on *in vitro* studies with cell cultures and to a lesser extent from *in vivo* administration. The generally accepted interactions fall into four categories: 1. Exchange of materials such as lipids and proteins between liposomes and the biological environment. 2. Adsorption or binding of liposomes to cells. 3. Cellular internalization by endocytosis or

phagocytosis, and 4. Rarely fusion of bound liposomes with cell membranes. *In vivo*, the majority of liposomes are taken up by the MPS cells through endocytosis and phagocytosis. The few escaping distribute into tissues and eventually disintegrate into individual components. Thus the fate of encapsulated drug is to be degraded within endosomes and lysosomes although a few can survive and be released back into the blood. i.e. doxorubicin and amphotericin B. Thus MPS uptake has proven to be a major barrier to liposomal delivery.

Sterically stabilized liposomes exhibit reduced macrophage uptake in tissue culture studies. The actual mechanism appears to involve reduction in protein adsorption and reduced cellular interactions. In fact, combination of PEG-PE and antibodies covalently attached to the surface of liposomes reduces antibody binding to cell surfaces expressing the specific antigen according to the PEG molecular weight and mole% as compared with similar liposomes lacking the PEG-PE (Mori et al., 1991; Park et al., 1995). With reduced MPS uptake and prolonged circulation, sterically stabilized liposomes have overcome the rapid inactivation of the encapsulated drugs found with conventional liposomes. They also reduce the potential for toxicity of MPS tissues by encapsulated drugs. Consequently, sterically stabilized liposomes provide opportunities to bypass rapid degradation or excretion of therapeutic agents from the blood.

### 2.3.7 THE SURFACE OF LONG CIRCULATING LIPOSOMES

The success in developing long-circulating liposomal preparations is due to liposome surface modification with glycolipids and polymers (Woodle, 1995). Despite the differences in chemical structures between, for example, GM1 (Gangliomonosides 1) and PEG derivatives, liposomes modified with these substances have one common feature: a brush' of elongated hydrophilic on all eukaryotic cell surfaces, and on surfaces (interfaces) formed by the extracellular matrix (Roberts and Mecham, 1993). The natural molecular brushes, which consist of a variety of glycoproteins and glycolipids, have an informational function (Roberts and Mecham, 1993) and a protective function. Thus, the protective' function would relate to the steric effects hindering liposome interactions with biological milieu, while the informational' function would relate to the interactions of the brush itself

(e.g., glycolipid recognition and binding by natural lectins) that can alter the overall biological reaction to the liposome. Obviously, the desired minimization of the overall biological reactivity of liposome requires minimization of brush reactivity and maximization of the protective properties. The major problem, however, relates to the facts that (a) the intimate mechanisms of steric hindrance remain largely obscure, and (b) there are no rational ways of minimizing brush interactions *in vivo* beyond the limit set by the current state of knowledge on macromolecule interactions *in vivo*. It has been shown that macromolecule and surface-grafted hydrophilic polymer molecules create relatively diffuse boundary layers. While their 'foots' are attached to the foundation, the rest of the chain retains freedom of movement and, therefore, remains 'dissolved' and randomly coils and moves around the attachment point (Milner, 1991). The resultant constantly changing conformation has been referred to as a 'mushroom' or a 'molecular cloud'. Multiple molecules form a brush. Hypothetically, molecular brushes sterically protect their foundation from interactions with a biological substrate, thus diminishing the overall reaction with the biological system. To date, this hypothesis has no reasonable alternatives. However, knowledge of the molecular brush action *in vivo* has a fragmentary character. The most commonly cited hypothesis suggests that polymer brushes sterically stabilize liposomes or other particles (Gabizon et al., 1988) or diminish opsonization (Lasic et al., 1991a). The suggested mechanisms include (i) 'repulsion' resulting from polymer chain constriction (ii) enhanced binding of 'dysopsonins' (plasma components that hypothetically prevent the recognition and uptake by macrophages) (Moghimi et al., 1993) (iii) difficulties in modeling antibodies around a flexible polymer molecule by the immune system (iv) formation of a 'molecular cloud' which shields the surface from opsonins (Torchilin and Papisov, 1994) (v) formation of a 'hydrated shell' that prevents opsonin entrance (Bogdanov et al., 1993), etc. Despite the variety of approaches to the explanation of the protective properties and the selected terms, all these hypotheses do not contradict each other and relate to different aspects of the same mechanism. Identifying brush parameters that show even rough

correlations with protective properties *in vitro* and *in vivo* would be highly valuable for liposome surface engineering.

### 2.3.7.1 Molecular brush: Structure and protective properties

According to the proposed mechanisms, steric shielding and steric obstruction depend on fundamentally different structural characteristics of the molecular brush. The efficacy of steric shielding depends on the probability of polymer chain interference with the surface – substrate collision. Therefore, steric shielding is expected to increase with concentration of brush material near the liposome surface, so both graft density and brush length can increase the efficacy of steric shielding. Some data on the connections between polymer length and the efficacy of steric protection is available (Mori et al., 1991). On the other hand, steric shielding depends on the substrate molecule size. Small molecules readily penetrate the brush, while larger molecules meet significant resistance (Papisov et al., 1995) Steric shielding should also correlate with the uniformity of the dynamic 'molecular cloud' formed by the brush over the surface. Conformational mobility of the brush results in higher cloud density and uniformity, which can enhance the shielding effect (Torchilin and Papisov, 1994). In model experiments, poly (ethylene glycol) grafted to liposomes decelerated protein interaction with the liposome surface, whereas liposome-grafted dextran (a more rigid polymer) did not affect protein-to-liposome interaction (Torchilin et al., 1994). A similar experiment conducted using graft copolymers of dextran showed, however, that dextran also may provide effective steric protection. Steric obstruction is expected to increase with brush density, rigidity and length. Cooperative 'non-specific' liposomesubstrate interactions, e.g. as in liposome cell surface or liposomeeellular matrix interactions, should be extremely sensitive to steric obstruction (Papisov et al., 1996). In general, dense brushes can be expected to have greater protective properties with the possible presence of a critical density. On the other hand, dense brushes can hypothetically have a greater intrinsic capability of non-specific binding (due to the presence of a larger number of polymer domains per surface square); therefore, the existence of a certain optimal brush density interval can be expected. Besides, polymer modified liposomes become unstable if a certain

brush density limit has been exceeded (Lasic et al., 1991). According to the modeling results, the overall biological effect of molecular brushes depends on the interplay of interactions and steric effects of all types. In turn, the latter depend on both brush structure and carrier type.

### 2.3.7.2 Interactions of protective polymers with components of biological systems

Besides the protective properties, discussed above, materials for liposome modification should meet another obvious requirement: their own interactions with biological milieu should be minimal. In other words, they should belong to the group of long-circulating polymers. Poly (ethylene glycol) (PEG) demonstrates, apparently, the best currently known combination of properties that provide long liposome circulation. The advantages of PEG and other low-reactive long-circulating polymers are utilized via assembling brush-like interfaces or various 'coatings' around liposomes and other drug carriers (Gref et al., 1995; Zalipsky, 1995). The currently available materials and methods for liposome surface modifications are reviewed elsewhere (Woodle and Lasic., 1992).

The main feature of long-circulating polymers can be defined as minimal presence of biologically reactive functional groups on their interface with the liquid phase. The term biologically reactive' refers here to polymer ability to form chemical bonds and to participate in either high- or low-energy interactions of any type with any components of biological systems. As in the case of the liposome surface, it is convenient to discuss the high-affinity (specific) and non-specific interactions separately. Polymer fragments and functional groups capable of interactions with recognition proteins may demonstrate potent effects on circulation via opsonization and increase in vascular permeability. For this reason, the biokinetics of liposomes modified by such polymers would be more vulnerable to changes caused by pathological processes (e.g., increased vascular permeability and appearance of acute-phase proteins in blood). Several macromolecules of biological origin and their components are known to interact with recognition proteins. Antigens interact with complementary immunoglobulins; several carbohydrates interact with a variety of recognition proteins (lectins); hydroxyl and amino groups may bind C3 and

C4, respectively. Carbohydrates are recognized by several receptors, including receptors present in large numbers in liver (to name a few, receptors binding galactose, mannose and fucose derivatives (Rice and Lee, 1990; Haltiwanger et al., 1986). Scavenger receptors of phagocytes may bind a variety of compounds (Rohrer, 1990). Various receptors bind peptides and several other compounds. Although receptor concentration in tissues (except RES) appears to be too low to affect polymer circulation at all but the smallest doses, polymer cross-reactivity with such receptors may cause various side effects and should, in general, be avoided. Dextran is considered to be one of the most biologically inert biopolymers (Larsen, 1989; Jeanes, 1986). Most of the other polysaccharides contain receptor-recognizable sites (Young and Leon, 1987; Wright et al., 1989), which hardly make them useful for carrier modification. Most synthetic polymers lack any receptor- and opsonin-binding groups. However, all functional groups without any exceptions are capable of cooperative interactions *in vivo*, for example based on Vander Waals, hydrophobic, dipoledipole, electrostatic interactions and formation of hydrogen bonds. Polymers containing several charged groups or hydrophobic sites are most reactive *in vivo*; the longest circulation times were reported for neutral or slightly negatively charged polymers. Uncharged hydrophilic modified by oligosaccharides, is due to saturation of functional groups with low nucleophilicity appear to be suitable for liposome protection. Because the components of any molecular brush are not completely inert, they can interact with the biological substrate via systems of collective weak associations. Obviously, long polymer chains and dense brushes are more vulnerable to cooperative interactions because of the presence of a greater number of groups forming weak bonds. There are indications that excessively long brushes (PEG, >5 kDa) are in fact inferior in their ability to prolong circulation time (Bedu-Addo and Huang, 1995). Graft copolymers of dextran and dextran-modified nanoparticles, unlike dextran, are readily recognized by some phagocyte populations and accumulate in lymph nodes after intravenous administration (Papisov and Weissleder, 1996; Papisov et al., 1995). Based on the above, the brush should be as short and loose as possible, regardless of the polymer used. This obviously confronts the requirements

directed to optimizing the protective properties of the same brush. Therefore, an optimal brush density providing minimal overall liposome interactions should exist and can be determined experimentally for each polymer type and liposome composition.

#### 2.3.7.3 Approaches to the development of better liposome surface modifiers

Considering the intrinsic vulnerability of polymers to cooperative interactions, the question remains: how the natural supramolecular and macromolecular structures (blood cells, plasma proteins) can remain in the circulation for weeks and months, if their components cannot avoid cooperative interactions? The answer would be undoubtedly important for liposome development: possibly, the mechanisms involved in this long-term retention could be utilized for developing ultra-long circulating liposomal drug carriers with exceptional target specificity. Although there is no direct experimental evidence, it seems possible that long circulation of natural cells and proteins, which are well known to be heavily modified by oligosaccharides, is due to saturation of the potential sites of non-specific binding. The total effective amount of circulating oligosaccharides consisting of structurally similar fragments is so high that all potential sites of non-specific binding must be occupied (while high-affinity centers specifically recognizing certain structural domains of the oligosaccharides remain vacant). If the above hypothesis is correct, polymers assembled of fragments structurally similar to those of carbohydrates (but lacking any receptor- and opsonins-recognizable domains) should be most resistant to non-specific binding, because all possible binding sites are already occupied by natural glycoconjugates. Obviously, it is important to avoid even the slightest cross-reactivity with recognition proteins and receptors, because cooperative weak interactions with several binding centers may still result in polymer recognition. Structures common for oligosaccharides include, in general, aliphatic secondary and primary OH groups, short carbon chains, and aliphatic acetal groups (in general, aliphatic acetal carbon and ether type oxygen). The structure of the most long-circulating' sialated oligosaccharides also contains *N*-acetyl groups. Poly(hydroxymethylethylene hydroxymethylformal) or Fleximer' was tested as a protective brush

component in a graft copolymer and nanoparticle models. The results have shown that Fleximer prolongs circulation of the model carriers much better than dextran (one of the most biologically inert polysaccharides). The observed circulation times (half-life >25 h) and biodistribution were similar (or better) to those of analogous polyethyleneglycol derivatives. The effect of fleximer on liposome circulation was similar. Another important factor of liposome viability relates to the stability of the protective brush (as well as the liposome itself). Due to a variety of interactions, the protective polymer can be chemically altered or removed from the liposome surface. The composition of the underlying lipid bilayer can also be altered as a result of lipid exchange, which may cause, in turn, significant changes in the protective brush. *In vivo*, such alterations are well known (Gattegno et al., 1979; Zocchi et al., 1987) and can be 'repaired' by cells, for example via production of new membrane components and either endocytosis or exocytosis ('shedding') the 'weared' ones (Comfurius et al., 1990). These phenomena are being studied and will probably provide valuable new approaches for drug carrier development.

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## 2.4 PROFILE OF CYCLOSPORINE: (CsA)

### 2.4.1 INTRODUCTION

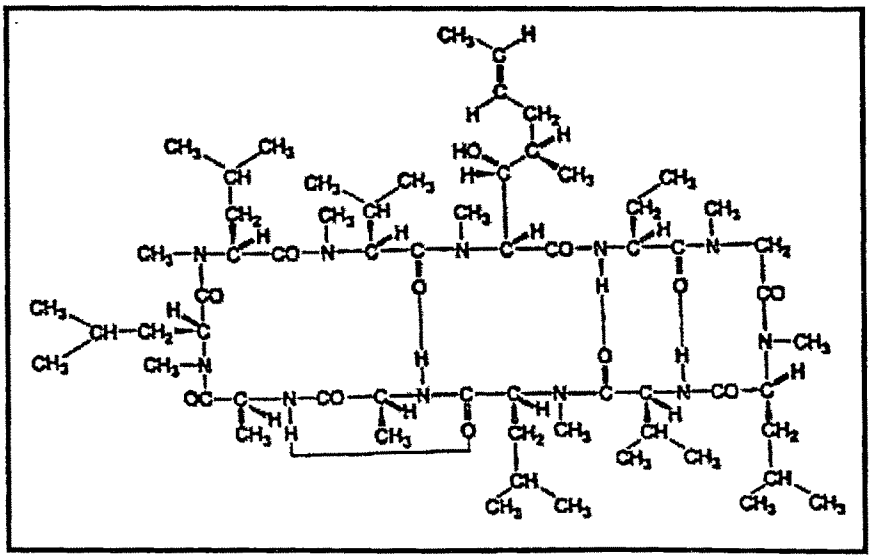
Cyclosporine is a lipophilic cyclic polypeptide immunosuppressant that interferes with the activity of T cells chiefly via calcineurin inhibition. It is a potent immunosuppressive agent extensively employed to avert graft rejection in kidney, bone and bone marrow transplant patients and to delay or prevent disease progression in patients with autoimmune disease and inflammation.

### 2.4.2 CHEMISTRY

#### 2.4.2.1 Structural formula

Cyclosporine belongs to a family of cyclic polypeptides derived from the fungus Tolypocladium inflatum Gams. It consists of 11 aminoacid residues (including a unique 9-carbon acid in position 1) arranged in a cyclic structure as shown below. All amide nitrogens are either hydrogen bonded or methylated. Cyclosporine contains a single D-amino acid residue in position 8, and the methylamide between residues 9 and 10 is in the cis configuration, all other methylamide moieties are in the trans form. Cyclosporine is lipophilic and very hydrophobic with a pKa of 2.9.

#### 2.4.2.2 Structure of Cyclosporine



### 2.4.3 PHARMACOLOGY

**2.4.3.1 Mechanism of action** (Goodman and Gilman)

Cyclosporine has a very selective inhibitory effect on T lymphocytes, suppressing the early cellular response to antigenic and regulatory stimuli. The immunosuppressive effect occurs following the heterodimeric complex consisting of cyclosporine bound to a cytoplasmic receptor protein, cyclophilin. The complex then binds to calcineurin, inhibiting the $Ca^{2+}$-stimulated serine/threonine phosphatase activity critical for dephosphorylation of cytosolic regulatory proteins, which, after removal of phosphate, are translocated to the nucleus to serve as subunits of transcription factor complexes (Schreiber, 1992). T-cell activation leads to enhanced transcription of a number of T-cell genes coding for specific cytokines, particularly interleukin-2 (IL-2), certain protooncogenes (e.g., c-myc and H-Ras), and selected cytokine receptors (e.g. IL-2 receptor). The consequence of cyclosporine binding to its receptor, cyclophilin, is the inhibition of calcineurin pathway and suppression of calcineurin activated events. Cyclosporine also can attenuate IL-2 production through increased expression of transforming growth factor β (TGF-β) which is a potent inhibitor of IL-2 stimulated T-cell proliferation and of generation of antigen-specific cytotoxic T-lymphocytes. Increased expression of TGF-β may contribute to the overall immunosuppressive effect produced by cyclosporine (Suthanthiran and Strom, 1994; Wiederrecht et al, 1993)

### 2.4.3.2 Drug disposition and pharmacokinetics

Cyclosporine can be administered intravenously as a 50mg/ml solution made up in ethanol-polyoxyethylated castor oil mixture. It also can be administered orally as a 25- or 100-mg soft gelatin capsule (SANDIMMUNE) or as a newer oral microemulsion formulation (SANDIMMUNE NEORAL). Cyclosporine administered in the original soft gelatin capsule formation is absorbed slowly and incompletely with bioavailability ranging from 20% to 50%. The microemulsion formulation was developed to improve the absorption characteristics of cyclosporine and was approved for clinical use in the United States in 1995. The bioavailabity of cyclosporine in the microemulsion formulation is improved over that in the soft gelatin capsule, clinical trials with SANDIMMUNE NEORAL in healthy volunteers and in stable renal transplant patients have demonstrated a reduction in the inter-

and intrapatient variability of all pharmacokinetic parameters (Kovarik et al, 1993; Mueller et al, 1994a).

Peak levels of cyclosporine in the plasma usually occur from 1.3 to 4 hours after oral administration (Kovarik et al, 1993). Ingestion of a fatty meal significantly delays absorption of cyclosporine in the gelatin capsule formulation but not that in the microemulsion formulation; this difference has important implications for individualizing dosage regimens for out-patient administration (Mueller et. al, 1994b) following access to the circulation by either oral or intravenous route, the drug distribute widely, as is suggested by a relatively large apparent volume of distribution (13 liters/kg). In whole blood, 50% to 60% of cyclosporine accumulates in erythrocytes. Leucocytes also avidly accumulate this drug, with 10% to 20% of the total amount of drug in the circulation being associated with the relatively small leukocyte fraction. This is due to the high cyclophilin content of these cells. The remainder of drug in the circulation is associated with the plasma lipoproteins. Cyclosporine disappears from the circulation with an elimination half-life of approximately 6 hours.

Cyclosporine is metabolized extensively in the liver to more than 30 metabolites, although there is considerable interindividual variation in the drug metabolism (Fahr, 1993; Christians and Sewing, 1993). The cyclic peptide structure of cyclosporine is relatively resistant to metabolism, but the side chains are extensively metabolized largely by the cytochrome P450 3A system. While metabolism is thought to result in inactivation of the immunosuppressive properties of cyclosporine, it is possible that some of the cyclosporine metabolites may contribute to immunosuppression or toxicity. Cyclosporine and its metabolites are excreted mainly through the bile into the feces; approximately 6% is excreted in the urine. In the presence of hepatic dysfunction, adjustments in dose may be necessary.

### 2.4.3.3 Cellular mechanism of action of cyclosporine (figure 2.4)

Cyclosporine readily diffuses into the cytoplasm of target cells. Among the pathways they inhibit are the T cell receptor-activated signal transduction pathway. Activation of the T-cell receptor causes, among other sequences, an increase in intracellular $Ca^{2+}$, which activates a $Ca^{2+}$ dependent, serine-

threonine phosphatase, known as calcineurin. One substrate of calcineurin, a cytosolic component of NFAT (NFATc; nuclear factor of activated T cells) moves from the cytoplasm to the nucleus upon dephosphorylation. By associating with other nuclear components of NFAT(NFATn), this calcineurin substrate regulates the transcription of many genes, including the genes encoding IL-2, granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor α (TNF- α), interferon α, and other interleukins. Calcineurin dephosphorylates another cytosolic regulatory protein, octamer activating protein (OAP), which also requires dephosphorylation for transport into the nucleus to serve as a transcription factor. Cyclosporine in association with its binding protein cyclophilin, stably associate with calcineurin at the endofacial surface of T cells and inhibit calcineurin catalytic activity and as a consequence, the nuclear translocation of NFAT and OAP. The CD3 glycoprotein is in close proximity to the antigen recognition T cell receptor. Antibodies directed against CD3 (e.g. muromonad CD3) block access of antigen to the T cell receptor, forming the basis for the use of muromonad CD3 as an immunosuppressive agent. MHC, major histocompatibilty complex; CAM, calmodulin.

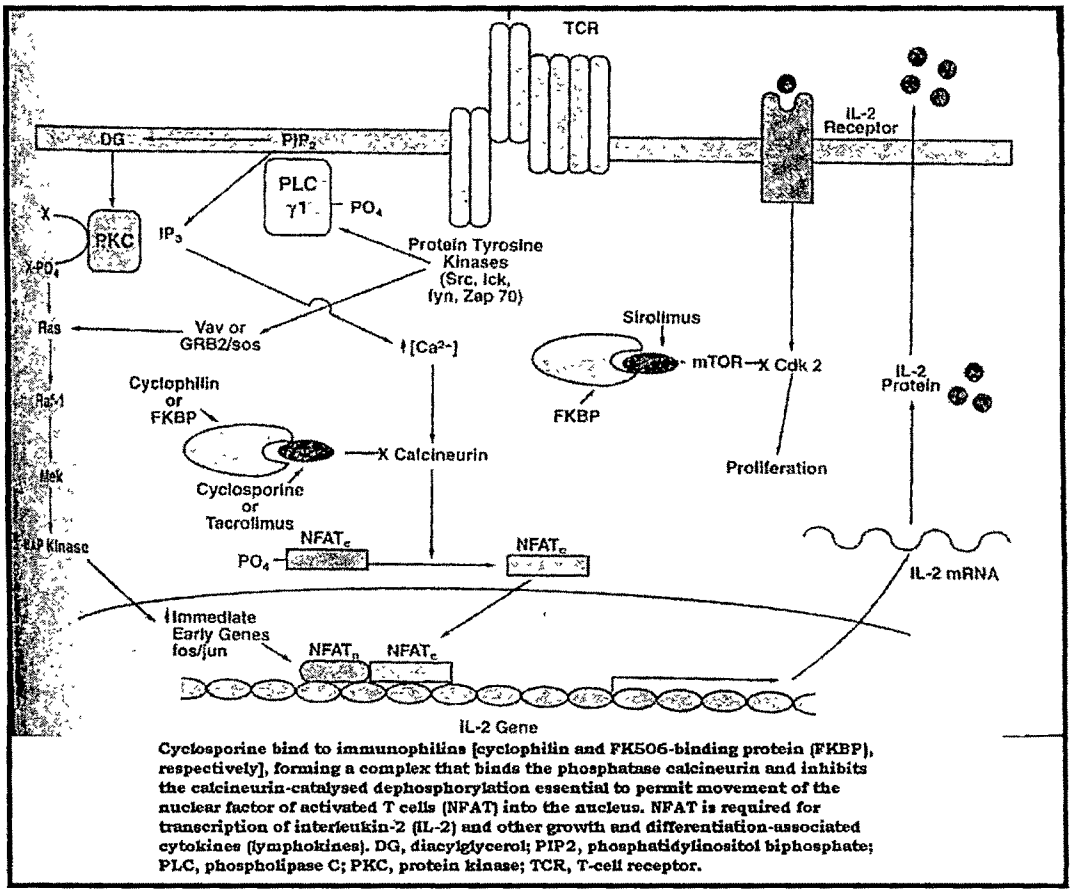
**2.4.3.4 Therapeutic uses**

Cyclosporine is one of the key drugs used to obtain immunosuppression necessary for the prevention and treatment of transplant rejection. It is used routinely together with other immunosuppressive agents (most often corticosteroids) in the transplantation of kidney, heart, and liver as well as increasingly in the transplantation of other organs, such as bone marrow, lung, heart/lung, and pancreas (Burke et al., 1994). It is also used as an antifungal agent.

Cyclosporine also has therapeutic value in a number of disorders where dysfunction of immunoregulation is thought to be an etiological factor (Faulds et al., 1993). Cyclosporine has been shown to be an effective therapy in acute ocular Behcets syndrome, endogenous uveitis, psoriasis, atopic dermatitis, rheumatoid arthritis, active Crohns disease and nephritic syndrome. In many of these cases, cyclosporine is combined with a corticosteroid. In addition, cyclosporine has been used as first line

treatment in patients with moderate or severe aplastic anaemia who are ineligible for bone marrow transplantation. Recent data have suggested a possible role in primary cirrhosis.

**Figure 2.4 Cellular Mechanism of Action of Cyclosporine**




**Cyclosporine bind to immunophilins [cyclophilin and FK506-binding protein (FKBP), respectively], forming a complex that binds the phosphatase calcineurin and inhibits the calcineurin-catalysed dephosphorylation essential to permit movement of the nuclear factor of activated T cells (NFAT) into the nucleus. NFAT is required for transcription of interleukin-2 (IL-2) and other growth and differentiation-associated cytokines (lymphokines). DG, diacylglycerol; PIP2, phosphatidylinositol biphosphate; PLC, phospholipase C; PKC, protein kinase; TCR, T-cell receptor.**

### 2.4.3.5 Dosage and administration

Oral treatment is initiated 4 to 24 hours prior to transplantation with a dose of 15mg/kg; this dose (given once daily) is continued for 1-2 weeks post operatively. Thereafter, the dosage is reduced each week until a maintenance dose of 3 to 10mg/kg per day is reached. Dosage is generally guided by signs of renal toxicity as judged from changes in creatinine clearance.

### 2.4.3.6 Toxicity

The major adverse effect of cyclosporine is renal toxicity. Nephrotoxicity can occur in as many as 75% of patients being treated with cyclosporine. It is frequently one of the major factors responsible for cessation or modification of therapy (Burke et al., 1994). Other toxicities include hypertension,

hepatotoxicity, neurotoxicity, hirsutism, gingival hyperplasia and gastrointestinal toxicity (nausea, vomiting, diarhoea, anorexia and abdominal pain).

**2.4.3.7 Drug interactions**

Cyclosporine interacts with a variety of commonly used drugs. The mechanisms responsible for many of these interactions are unclear. The clearance of cyclosporine is accelerated with coadministration of Phenobarbital, phenytoin, trimethoprim-sulfamethoxazole, and rifampin, largely as a result of induction of the hepatic P450 system. This interaction has produced decreased cyclosporine levels, resulting in rejection of transplanted organs. The clearance of cyclosporine is decreased when it is coadministered with amphotericin B, erythromycin, or ketoconazole. This interaction has resulted in an increased risk of cyclosporine toxicity. These interactions are shown in various reports (Campana et al., 1996; Mezzano et al., 1998; Asberg, et al., 1999; Sud et al., 1998; Sagedal et al., 1998; Maltz et al., 1999).

**2.4.3.8 Precautions**

Patients with malabsorption may have difficulty in achieving therapeutic levels with cyclosporine. Hypertension is a common side effect of cyclosporine therapy. Mild or moderate hypertension is more frequently encountered than severe hypertension and the incidence decreases over time. Antihypertensive therapy may be required. Control of blood pressure can be accomplished with any of the common antihypertensive agents. However, since cyclosporine may cause hyperkalemia, potassium-sparing diuretics should not be used. While calcium antagonists can be effective agents in treating cyclosporine-associated hypertension, care should be taken since interference with cyclosporine metabolism may require a dosage adjustment.

**2.4.3.9 Laboratory tests**

Renal and liver functions should be assessed repeatedly by measurement of BUN, serum creatinine, serum bilirubin, and liver enzymes.

### 2.4.4.10 Adverse reactions

1. The principal adverse reactions of cyclosporine therapy are renal dysfunction, tremor, hirsutism, hypertension, and gum hyperplasia.

2. Hypertension, which is usually mild to moderate, may occur in approximately 50% of patients following renal transplantation and in most cardiac transplant patients.

3. Glomerular capillary thrombosis has been found in patients treated with cyclosporine and may progress to graft failure.

4. Hypomagnesemia has been reported in some, but not all, patients exhibiting convulsions while on cyclosporine therapy. Although magnesium-depletion studies in normal subjects suggest that hypomagnesemia is associated with neurologic disorders, multiple factors, including hypertension, high dose methylprednisolone, hypocholesterolemia, and nephrotoxicity associated with high plasma concentrations of cyclosporine appear to be related to the neurological manifestations of cyclosporine toxicity.

5. The following reactions occurred in 2% or less of patients: allergic reactions, anemia, anorexia, confusion, conjunctivitis, edema, fever, brittle fingernails, gastritis, hearing loss, hiccups, hyperglycemia, muscle pain, peptic ulcer, thromboytopenia and tinnitus.

6. The following reactions occurred rarely: anxiety, chest pain, constipation, depression, hair breaking, hematuria, joint pain, lethargy, mouth sores, myocardial infarction, night sweats, pancreatitis, pruritus, swallowing difficulty, tingling, upper GI bleeding, visual disturbance, weakness, weight loss.

## 2.4.4 QUATITATIVE ANALYTICAL PROFILE OF CYCLOSPORINE

### 2.4.4.1 Chromatographic techniques

Various HPLC methods are available for the estimation of cyclosporine, which is tabulated below (table 2.3). HPLC based methods are useful for estimation in biological fluids and in dosage form using UV detector ranging from 200–218 nm.

**Table 2.3 Summary of HPLC methods for analysis of cyclosporine**

| Column used | Mobile phase (flow rate) | Detection | Medium | Reference |
|---|---|---|---|---|
| Waters $C_{18}$ column (dimensions not given). | acetonitrile/$H_2O$ (7:3)(0.8 ml/min) | 210 nm | Biological fluids | McBride et al., 1995 |
| Microbore reversed-phase column | methanol/water/acetonitrile (4:7:10) | 205nm | Biological fluids | Brozmanova et al., 2000 |
| RESOLVE $C_{18}$ Column (20 cm × 4.6 mm i.d.) | Aqueous 80% acetonitrile (1ml/min) | 208 nm | Blood | Tang et al., 2000 |
| Nova-Pak Phenyl column (15 cm ×3.9 mm i.d.) | methanol/CAN /$H_2O$ (17:17:16) (1ml/min) | 210 nm | Urine and Plasma | Van Tellingen et al., 1998 |
| 5m Spherisorb S5 column. (25 cm × 4.6 mm i.d.) | propan-2-ol/hexane (1:9) (1.45 ml/min) | 212 nm | Biological fluids | Khoschsorur et al., 1997 |
| 5mSi 60 column (2.5 cm ×4 mm i.d.) | hexane/ethanol (17:3) (1.5 ml/min) | 210 nm | Whole blood | Poirier et al., 1994 |
| Millipore-Waters C18 Bondapak column (3.9 ×300 mm i.d.) | methanol/H2O (81:19) (1.3 ml/min) | 486 nm. | Whole blood | Maguire et al., 1993. |
| Micropak Si-5 (40 cm ×4 mm) C18-10 | n-hexane: isopropanol: methanol (8:1:1) (1 ml/ min) | 215 nm. | Whole blood | Tao et al., 1993. |

### 2.4.4.2 Other methods

**Mass spectrophotemetry** (Muddiman et al., 1994)

Cyclosporin A) was determined in real or synthetic blood by TOF SIMS and MALDI-TOF MS. Blood (1 ml) was mixed with 50l of methanolic cyclosporin D internal standard (625ng/ml), diluted with $H_2O$ and extracted with ether. The organic extract was evaporated to dryness, the residue was dissolved in methanol and the solution was mixed with 25mM-HCl and hexane. The aqueous layer was mixed with 25mM-NaOH and ether, the organic layer was evaporated to dryness and the residue was dissolved in 150l of methanol. A 3l portion of the solution was and analysed using a TOF SIMS III instrument with an EI ion source, with detection by a channel plate-scintillator-photomultiplier combination. For MALDI-TOF MS, 4-8l of extract was mixed 1:1 with 2, 5-dihydroxybenzoic acid matrix solution [20 mg/ml in $H_2O$/methanol (1:1)] and a 1l portion was deposited on a stainless-steel substrate and dried. Analysis was performed using a modified LAMMA 1000 mass spectrometer having a $N_2$ laser. The detection limits were 7 and 10 ng/ml of **I** for TOF SIMS and MALDI-TOF MS, respectively. The RSD were 3-5% and 4-8% for the two methods, respectively. The results agreed with those obtained by HPLC.

**Enzyme-Multiplied Immunoassay Technique (EMIT)** (Angeles de Cos et al, 1998).

Cyclosporine was determined in whole blood samples by EMIT using EMIT 2000 Cyclosporine Specific Assay kits (Behring Diagnostics Inc.) on a Cobas-Fara analyzer (Roche Diagnostic Systems) and by FPIA using AxSym Cyclosporine Monoclonal Whole Blood FPIA kits on an AxSym analyzer (Abbott Laboratories), both carried out according to the manufacturers instructions. Useful calibration ranges were up to 500 and 800 ng/ml of cyclosporin for EMIT and FPIA, respectively, and limits of quantitation were 12-31 ng/ml and 14 ng/ml, respectively. Intra- and inter-day RSD were similar for both assays (<6.5 and <7%, respectively). Good correlation was observed between the two methods ($r$ = 0.97) with FPIA on the AxSym producing an average 10% overestimation compared with EMIT. The methods were compared in a study of cyclosporin whole blood

concentrations determined in samples from patients after heart, liver, bone marrow, lung and kidney transplantation.

**Gas Chromatography** (Wang et al., 2000)

Two capsules of cyclosporin A (**I**) were dissolved in DMSO then mixed with 1 ml 10% n-propanol (internal standard) in DMSO before diluting to 50 ml. Ethanol (**I**) and propylene glycol (**II**) in 2l of the solution were separated and determined by GC on a 2 m column packed with GDX-101, operated with temperature programming from 165°C (held for 12 min) to 280°C at 40°C/min and FID. The calibration graphs for **I** and **II** were linear from 2-6 and 1-3g, respectively. The recoveries were 99.9-101.4% with RSD of <1.1%.

**Radioimmunoassay** (Lee et al., 2000)

A significant overestimation of cyclosporin A (CsA) by a radioimmunoassay using $^{125}$I-labeled monoclonal antibody ($^{125}$I-RIA), compared to the reference HPLC method, has been reported for a limited number of samples from transplant patients. However, the extent of the discrepancy, with respect to bioavailability parameters, has not been examined for the case of the oral administration of a single dose CsA to healthy subjects where a number of factors which might be involved in this overestimation (*e.g.* under steady state condition and a significant accumulation of CsA metabolites) would be absent. Therefore, the objective of this study was to assess the effect of potential difference manifested by the two analytical procedures, $^{125}$I-RIA and HPLC, on the bioavailability analysis of CsA. An oral CsA formulation was administered to 22 healthy male subjects and the blood samples were analysed by both $^{125}$I-RIA and HPLC. Significant discrepancies in the estimated CsA concentrations by the two methods (paired *t*-test, $P >> 0.001$) were found. The difference (bias) increased with increasing concentrations of blood CsA ($P >> 0.001$). However, despite the bias in CsA estimations, the AUC and $C_{max}$, obtained by $^{125}$I-RIA and HPLC methods showed only small differences (*i.e.* 2% for AUC and 7% for $C_{max}$). Thus, our results suggest that the bias of the $^{125}$I-RIA vis-a-vis the HPLC method in the estimation of CsA blood levels may not, in practice, affect the bioavailability analysis (*e.g* bioequivalence study) of CsA in a situation where a single dose CsA is orally administered to healthy subjects.

**Fluorescence polarization immunoassay (FPIA)** (Kurosaki et al., 1999)

A modified fluorescence polarization immunoassay method incorporating fat emulsion (E-FPIA) to determine cyclosporin A concentrations in rat skin. A modified version of the commercially available FPIA kit, TDX cyclosporine monoclonal whole blood, was used. The method was modified by incorporating the fat emulsion for intravenous infusion, Intralipos, to dissolved cyclosporin A extracted (with $CHCl_3$) from skin tissue and methanol/purified $H_2O$ (7:3) was used as a sample pretreatment instead of the precipitation reagent conventionally used. The detection limit was 25 ng/ml with reproducibility RSD <2%. The method should be useful for evaluating the topical pharmacokinetics of cyclosporin A in skin.

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## 2.5 PROFILE OF LEUPROLIDE ACETATE

### 2.5.1 INTRODUCTION

Leuprolide acetate is a potent Gonadotropin releasing hormone agonist (GnRH agonist) that stimulates the release of luteinising hormone. Prostate cancer is the most commonly diagnosed, and the second leading cause of death from cancer in North American and European men. Epithelial ovarian cancer is the most common cause of death from gynecologic malignancies in the United States. It is estimated that 21,000 cases of ovarian cancer were diagnosed and 13,000 deaths from this disease occurred in 1992 (Tetsu et al., 1994). Androgen deprivation is the only effective systemic therapy available for metastatic prostate cancer. The inability of androgen deprivation to completely and permanently eliminate all prostate cancer cell populations is manifested by the predictable pattern of initial response and relapse, with the ultimate progression to androgen independence. Androgen deprivation is associated with a gradual transition of prostate cancer cells through a spectrum of androgen-dependence, androgen sensitivity and ultimately androgen independence. There is mounting evidence supporting the concept that prostate cancer progression is accompanied by a shift in reliance on endocrine controls to paracrine and eventually autocrine controls and that this complex process is the result of changes, which occur at molecular levels of cellular control. However, the molecular mechanisms involved in the development of androgen-independent prostate cancer are unknown. Historically, clonal selection and adaptation have been advanced to explain progression, but these general theories have been supplanted largely by molecular concepts related to the androgen receptor. Recognition of androgen receptor mutations, gene amplification, co-regulators and signal transduction crosstalk has given rise to the possibility of studying the primary events that trigger progression.

### 2.5.2 DESCRIPTION

#### 2.5.2.1 Molecular Formula

The chemical name is 5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide acetate (salt).

**2.5.2.2 Molecular weight** 1269.5

### 2.5.3 PHYSIOCHEMICAL PROPERTIES

**2.5.3.1 Solubility** Freely soluble in water, isopropyl alcohol and dimethyl sufoxide.

#### 2.5.3.2 Ionisation constant

Leuprolide acetate has three ionizable sites; the imidazolyl nitrogen of His (pKa=6.0), the phenolic hydroxyl of Tyr (pKa=10.0), and the guanidine nitrogen of Arg (pKa=13). Since the guanidine nitrogen is extremely basic, this peptide as synthesized exists in the protonated form and is generally associated with atleast 1 mole of acetic acid. The drug is a hydrophilic salt ($-NH_3.^-OOCCH_3$) because of the short hydrocarbon backbone of acetic acid. Leuprolide also is mostly ionized across a wide range of physiological interest, and is not absorbed orally. For this reason it was felt that salts with improved lipophilicity might be more bioavailable nasally or orally than the acetate form of the drug.

### 2.5.4 Clinical pharmacology

Leuprolide acetate, a nonapeptide is one of the GnRH superagonists most widely used in the treatment of sex hormone dependent tumors such as prostate carcinoma in men, breast cancer and ovarian cancer in women (Chrisp et al., 1991; Oesterling, 1991; Tunn et al., 1998; Garnick, 1984; Okada et al., 1983; Redding et al., 1981). GnRH agonists induce cell cycle arrest in the Go/G1 phase but the detailed molecular mechanism is unknown. One of the proposed mechanisms of the anti-tumor effect of GnRH agonists is the down regulation of GnRH receptor (GnRH-R) activity in the pituitary gland that results in inhibition of sex steroid secretion in patients with premenopausal breast cancer or prostate cancer. The other probable mechanism of action is the direct action of GnRH agonists via the GnRH-R, which is expressed in malignant tumors that differs from the pathway mediated via the pituitary. This direct action promotes the antiproliferative effect of these agents on the tumors (Nagai et al., 2002). GnRH-based system is expressed in prostate cancer, which might participate in the local regulation of tumor growth. This hypothesis has been later confirmed by showing that the activation of locally expressed GnRH-R by means of potent GnRH superagonists significantly reduces the

proliferation of prostate cancer cells, both *in vitro* and *in vivo* (Dondi et al., 1994; Limonta et al., 1992; Dondi et al., 1998). Reports revealed that the nucleotide sequence of GnRH receptors in human breast and ovarian tumors is identical with that found in pituitary (Schally, 1994; Loop et al., 1995; Qayum et al., 1990; Kakar et al., 1994).

The inhibitory action of GnRH agonist in endometrial, ovarian, breast, prostate and hepatic carcinoma cell lines has been suggested to occur through alteration of cell cycle progression, programmed cell death and the expression of growth factors and cytokines and their receptors (Mullen *et al.*, 1991; Thompson *et al.*, 1991; Connor *et al.*, 1994; Borri *et al.*, 1998; Mizutani *et al.*, 1998; Takeuchi *et al.*, 1998; Chegini, 2000; Imai and Tamaya, 2000). In ovarian and breast tumour cell lines, GnRH agonist is reported to arrest the cells in G0/G1 of the cell cycle, and is reported to enhance programmed cell death in endometrial carcinoma cells (Mullen *et al.*, 1991; Thompson *et al.*, 1991; Mizutani *et al.*, 1998, Grundker et al., 2001b). Leuprolide acetate inhibits DNA synthesis without affecting cell proliferation of LSMC and MSMC (leiomyoma amd myometrial smooth muscle cells), indicates a G0/G1 cell cycle arrest, as has been reported for LSMC (Mizutani *et al.*, 1998).

### 2.5.4.1 LHRH in prostate cancer

LHRH and LHRH receptors have been reported to be expressed also in some peripheral tissues, particularly in those related to the endocrine system. Investigations were carried out to found whether LHRH and LHRH receptors might be present in prostate cancer, and whether they might be involved in the control of tumor growth. These studies have been performed in human prostate cancer cell lines, either androgen-dependent (LNCaP) or androgen-independent (DU 145). By RT-PCR (Reverse transcriptase-polymerase chain reaction) the mRNA for LHRH is expressed in both LNCaP and DU 145 cells (Limonta *et al.* 1993; Dondi *et al.* 1994). In these cell lines the presence of the mRNA coding for the LHRH receptor, which appeared to be identical in its sequence to that found in the pituitary (Dondi *et al.* 1994). By Western blot analysis, and by using a specific monoclonal antibody raised against the human pituitary receptor (Karande *et al.* 1995), a protein band of approximately 64 kDa is present in membrane preparations of prostate

cancer cells. This molecular weight corresponds to that previously reported for the LHRH receptor at pituitary level in humans (Karande *et al.* 1995). Taken together, these data demonstrated that a LHRH-based system is expressed in prostate cancer, which might participate in the local regulation of tumor growth. This hypothesis has been later confirmed by showing that the activation of locally expressed LHRH receptors by means of potent LHRH superagonists significantly reduces the proliferation of prostate cancer cells, both *in vitro* (Limonta *et al.* 1992; Dondi *et al.*, 1994), and *in vivo* (Dondi *et al.* 1998).

On the basis of these results, in either androgen-dependent or androgen-independent prostate cancer, the local LHRH system might act as a paracrine/autocrine negative regulator of tumor growth. These observations are in agreement with those reported for tumors of the female reproductive tract, such as breast (Keri *et al.* 1991; Kakar *et al.* 1994; Kottler *et al.* 1997), endometrial (Imai *et al.* 1994; Chatzaki *et al.* 1996), and ovarian (Yin *et al.* 1998; Emons *et al.* 2000) cancer. These data seem to suggest that, when utilized for the treatment of hormone-related tumors, LHRH agonists might be different to that described for the pituitary receptor.

In preliminary experiments, the treatment of prostate cancer cells, either androgen-dependent or androgen-independent, with LHRH agonists does not affect phosphoinositide turnover or intracellular Ca++ levels. This suggested that, in these cells, LHRH receptors might not be coupled to the Gq/11-PLC system. Therefore, the antiproliferative action of LHRH agonists might be mediated by the Gi-cAMP signal transduction pathway. It is well known that pertussis toxin (PTX), through ADPribosylation of Gi proteins, impairs the receptor-effector interaction in this pathway (Ui and Katada, 1990). Therefore, studies were carried out to found, whether PTX might interfere with the antimitogenic action of a potent LHRH agonist (Zoladex, LHRH-A). As expected, LHRH-A significantly inhibited prostate cancer cell growth; the antiproliferative action of the LHRH agonist was completely prevented by PTX, thus suggesting that the receptor might be coupled to a Gi protein.

To verify this hypothesis, whether LHRH-A might affect PTX-induced ADP-ribosylation of this protein, the prostate cancer cell membranes were

incubated with PTX, in the presence of 32P-NAD, brought about ADP-ribosylation of a 41-kDa Gi protein. LHRH-A substantially counteracted the transfer of 32P ADPribose to this protein. To definitely confirm that, in prostate cancer cells, LHRH receptors might be coupled to Gi proteins, the effects of LHRH-A on forskolin-induced cAMP accumulation was studied; it is actually well known that the Gi subunit is negatively correlated with cAMP production. Hence, in tumor cells, LHRH-A did not affect cAMP levels when given alone. However, the LHRH analog completely counteracted the increase of cAMP levels induced by forskolin. Taken together, these results indicate that, in prostate cancer cells, the LHRH receptor is linked to a Gi protein, which, through inhibition of cAMP accumulation, may mediate the antiproliferative action of the peptide. These observations are in line with those reported for the LHRH receptor in ovarian and endometrial carcinoma (Imai et al., 1996; Imai and Tamaya 2000). The molecular mechanisms which follow Gi activation to lead to the inhibition of cancer cell proliferation are still poorly understood. In previous studies, reports showed that LHRH agonists counteract the mitogenic stimuli of growth factors, such as EGF or IGF (Moretti et al. 1996; Montagnani et al., 1999). These LHRH agonists seem to interfere with some of the intracellular events activated by the growth factors, such as receptor expression or receptor tyrosine phosphorylation (Moretti *et al.* 1996; Montagnani *et al.* 1999). It is then possible that, after the activation of the Gi protein, the reduced levels of cAMP might directly affect the intracellular mechanisms, which mediate mitogenic stimuli in cancer cells.

### 2.5.4.2 LHRH in melanoma

Van Groeninghen and coworkers (1998) reported that LHRH receptors might be expressed in melanoma cells. Cutaneous melanoma is a tumor known for its uncontrollable growth and for its ability to give rise to metastases (MacKie, 1998). The incidence of this tumor is increasing dramatically (Parkin et al., 1999) and, although its prognosis has improved in the last decades particularly due to early diagnosis, this remains very poor in advanced cases, when tumor cells acquire a strong potential to disseminate metastases (MacKie, 1998). Moreover, advanced melanoma is a multistep process, which starts from the initial transformation of

melanocytes, then goes through a radial growth phase, to eventually progress to the vertical growth phase (Lazar-Molnar et al., 2000). It is particularly in this phase that tumor cells start giving rise to metastases (Shih and Herlyn, 1993; Shih and Herlyn, 1994). The molecular mechanisms that are involved in the growth and progression of melanoma are still poorly understood. The experiments here described have been performed to clarify whether a LHRH-based system (LHRH and the respecitve receptors) is expressed in melanoma cells, whether the activation of this system might affect the proliferative rate as well as the metastatic properties of this tumor.

### 2.5.4.3 Expression of LHRH and LHRH receptors

The expression of both LHRH and LHRH receptors in melanoma cells has been investigated by RT-PCR, as described above for the studies performed in prostate cancer cells. With regard to the expression of LHRH, according to the sequence of the oligonucleotide primers used, the predicted fragment of 228 bp was observed in both BLM and Me15392 cells. After Southern blotting, the cDNA fragments hybridized with the 32P-labeled oligonucleotide probe specific for the LHRH cDNA (Oikawa et al., 1990). In the case of the expression of the LHRH receptor mRNA, the results obtained demonstrate that the predicted 885-bp cDNA fragment is present both in BLM and in Me15392 cells (melanoma cell lines); this band hybridized with the 32P-labeled probe specific for the LHRH receptor cDNA (Kakar et al., 1994). The presence of LHRH receptors in melanoma cells has been further investigated at the protein level, by Western blot and by using the specific F1G4 antibody raised against the human pituitary receptor (Karande et al., 1995). The results obtained demonstrate that a protein of approximately 64 kDa molecular mass is present in membrane preparations from both BLM and Me15392 cells. This molecular weight corresponds to that previously reported for the human pituitary receptor (Wormald *et al.*, 1985). Finally, LHRH receptors in melanoma cells have been analyzed also in terms of binding parameters. Radioreceptor assay have been performed by using 125I-LHRH-A as the specific ligand (Limonta et al., 1992; Dondi et al., 1994). The assays demonstrated that binding sites for 125I-LHRH-A are present on the membranes of both BLM and Me15392 cells. So far,

divergent results have been reported for the binding characteristics of LHRH receptors in cancers related to the reproductive tract (Limonta et al., 1992; Emons et al., 1993; Dondi et al. 1994; Emons and Schally 1994; Imai et al. 1994).

#### 2.5.4.4 Antiproliferative activity of LHRH

LHRH is expressed, together with its receptors, in hormone-related tumors, such as prostate cancer, to act as a local autocrine/paracrine growth inhibitory factor. In prostate cancer cells, the LHRH receptor is coupled to the Gi-cAMP pathway to inhibit cell proliferation. This is at variance with the LHRH receptor at pituitary level, which is coupled to the Gq-PLC pathway to stimulate gonadotropin synthesis and secretion.

LHRH and LHRH receptors are expressed also in tumors that are not classically related to the endocrine system, such as melanoma. In melanoma cells, the locally expressed LHRH-based system negatively regulates the proliferation of the cells as well as their ability to migrate towards chemotactic stimuli and to invade a reconstituted basement membrane. Therefore, in melanoma, the activation of LHRH receptors might reduce not only tumor growth but also its metastatic potential. Moreover, the LHRH receptor might represent a new diagnostic (and possibly prognostic) marker for thedetection of skin tumors.

#### 2.5.4.5 Uses

Leuprorelin acetate, a GnRH agonist, acts as a potent inhibitor of gonadotropin secretion when given on a continuous basis and in therapeutic doses. Animal and human studies indicate that following an initial stimulation, chronic administration of leuprorelin acetate results in suppression of ovarian and testicular steroidogenesis. This effect is reversible on discontinuation of therapy. Administration of leuprorelin acetate causes inhibition of the growth of certain hormone dependent tumours (prostatic tumours in Nobel and Dunning male rats and DMBA-induced mammary tumours in female rats) as well as atrophy of the reproductive organs.

**2.5.4.6 Pharmacokinetics** (Tunn et al., 1998; Sennello et al., 1986)

Leuprorelin acetate is not active when given orally. Bioavailability of this agent following subcutaneous administration is comparable to that after intramuscular administration. Following a single administration of Leuprorelin Acetate Depot Suspension 3-Month (11.25mg), a rapid increase of leuprorelin acetate concentration was observed. A mean peak leuprorelin plasma concentration of 21.82(± 11.24)ng/mL was observed three hours after injection. Leuprorelin acetate reached plateau levels within 7 to 14 days after injection. At week 4, a mean leuprorelin plasma concentration of 0.26(± 0.10)ng/mL was noted. It then declined to a mean leuprorelin plasma concentration of 0.17(± 0.08)ng/mL at 12 weeks.

**2.5.4.7 Distribution**

The mean steady-state volume of distribution of leuprorelin following intravenous bolus administration to healthy male volunteers was 27L. In vitro binding to human plasma proteins ranged from 43% to 49%.

**2.5.4.8 Metabolism**

In healthy male volunteers, a 1mg bolus of leuprorelin administered intravenously, revealed that the mean systemic clearance was 7.6 L/h, with a terminal elimination half-life of approximately three hours based on a two-compartment model. Animal studies have shown $^{14}C$-labelled leuprorelin was metabolised into smaller inactive peptides which may be further catabolised.

**2.5.4.9 Excretion**

Following administration of Leuprorelin Acetate for Depot Suspension 3.75mg to three patients, less than 5% of the dose was recovered as parent and M-1 metabolite in the urine over 27 days. The pharmacokinetics of the medicine in hepatic-and renal-impaired patients has not been determined.

**2.5.4.10 Indications**

Prostate Cancer

Leuprorelin Acetate for Depot Suspension 3-month (11.25mg) is indicated in the palliative treatment of advanced prostatic cancer.

Endometriosis

Leuprorelin Acetate for Depot Suspension 3-Month (11.25mg) is indicated in the treatment of endometriosis for a period of six months. It can be used as sole therapy or as an adjunct to surgery.

Uterine Fibroids

Leuprorelin Acetate for Depot Suspension 3-Month (11.25mg) is also indicated in the treatment of leiomyoma uteri (uterine fibroids) for a period up to six months. Therapy may be preoperative prior to myomectomy or hysterectomy, or it may provide symptomatic relief for the perimenopausal woman who does not desire surgery.

**2.5.4.11 Dosage and administration**

Lucrin Depot 3-month is a formulation of leuprorelin acetate supplied as sterile lyophilised microspheres in a vial which, when mixed with the accompanying diluent, forms a suspension. It is administered as an intramuscular or subcutaneous injection every three months.The single dose vial of Lucrin Depot contains leuprorelin acetate (11.25mg),a polymer, poly (DL-lactic acid) and D-mannitol. The ampoule of diluent contains carboxymethylcellulose sodium, D-mannitol, polysorbate 80 and Water for Injection USP.

Viadur™ (leuprolide acetate implant)

Viadur™ (leuprolide acetate implant) is a sterile nonbiodegradable, osmotically driven miniaturized implant designed to deliver leuprolide acetate for 12 months at a controlled rate. The system contains 65 mg of leuprolide (free base). Leuprolide acetate is a synthetic nonapeptide analog of naturally occurring gonadotropin-releasing hormone (GnRH or LH-RH). The analog possesses greater potency than the natural hormone.

Viadur™ contains 72 mg of leuprolide acetate (equivalent to 65 mg leuprolide free base) dissolved in 104 mg dimethyl sulfoxide. The 4 mm by 45 mm titanium alloy reservoir houses a polyurethane rate-controlling membrane, an elastomeric piston, and a polyethylene diffusion moderator. The reservoir also contains the osmotic tablets, which are not released with the drug formulation. The osmotic tablets are composed of sodium chloride, sodium carboxymethyl cellulose, povidone, magnesium stearate, and sterile

water for injection. Polyethylene glycol fills the space between the osmotic tablets and the reservoir. A minute amount of silicone medical fluid is used during manufacture as a lubricant. The weight of the implant is approximately 1.1g.

Leuprorelin Acetate for Depot Suspension 3-month (11.25mg) must be administered under the supervision of a physician. The recommended dose of Leuprorelin Acetate Depot Suspension 3-month is 11.25mg, administered as a single subcutaneous or intramuscular injection every three months. As with other medicines administered by injection, the injection sites should be varied periodically.

**2.5.4.12 Contraindications**

1. Lucrin Depot 3-month is contraindicated in patients with known hypersensitivity to leuprorelin acetate, similar nonapeptides, or any of the excipients. Isolated cases of anaphylaxis have been reported with the monthly formulation of leuprorelin acetate.

2. Leuprorelin Acetate is contraindicated in women who are, or may become pregnant while receiving the medicine. When administered on day six of pregnancy at test dosages of 0.00024, 0.0024 and 0.024mg/kg (1/600 to 1/6 of the human dose) to rabbits, it produced a dose-related increase in major foetal abnormalities. Similar studies in rats failed to demonstrate an increase in foetal malformations. There was increased foetal mortality and decreased foetal weights with the two higher doses of leuprorelin acetate in rabbits and with the highest dose in rats. The effects on foetal mortality are logical consequences of the alterations in hormonal levels brought about by this medicine. Therefore, the possibility exists that spontaneous abortion may occur if the medicine is administered during pregnancy. Leuprorelin acetate should not be administered to patients with undiagnosed vaginal bleeding.

**2.5.4.13 Adverse effects**

Side effects such as Angina, bradycardia, cardiac arrhythmia, congestive heart failure, ECG changes/ischemia, hypertension, hypotension, murmur, myocardial infarction, phlebitis, stroke, syncope/blackouts, tachycardia, thrombosis, varicose veins, constipation, diarrhoea, dry mouth, duodenal

ulcer, dysphagia, gastrointestinal bleeding, vomiting, diabetes, thyroid enlargement, delusions, depression, dizziness, hypesthesia, insomnia, lethargy, libido increased, lightheadedness, memory disorder, mood swings, nervousness, neuromuscular disorders, numbness, paraesthesia, peripheral neuropathy and sleep disorders. Other effects include Cough, dyspnoea, epistaxis, haemoptysis, pharyngitis, pleural effusion, pleural rub, pneumonia, pulmonary fibrosis, pulmonary infiltrate, respiratory disorders, sinus congestion. There have been very rare reports of suicidal ideation and attempt. Increase in transaminase values and triglycerides.

**2.5.4.14 Overdosage**

There is no clinical experience with the effects of an acute overdosage of leuprorelin acetate depot suspension. In animal studies, doses of up to 500 times the recommended human dose resulted in dyspnoea, decreased activity and local irritation at the injection site. In cases of overdosage, the patients should be monitored closely and management should be symptomatic and supportive.

**2.5.4.15 Pharmaceutical precautions**

Store below 25°C. Refrigeration is not necessary. Do not freeze.

**2.5.5 QUANTITATIVE METHODS** include ultraviolet spectroscopy, HPLC, Radioimmunoassay, Enzyme linked immunosorbent assay and mass spectrometry.

**2.5.5.1 HPLC**

Various HPLC methods are available for the estimation of leuprolide acetate, which is tabulated below (table 2.4). HPLC based methods are useful for estimation in biological fluids and in dosage form using UV detector ranging from 200–250nm.

**Table 2.4 Summary of HPLC methods for analysis of leuprolide acetate**

| **Column used** | **Mobile phase (flow rate)** | **Detection** | **Medium** | **Reference** |
|---|---|---|---|---|
| $C_{18}$ MICROSORB-MV column (15 cm × 4.6 mm i.d.) as mobile phase | 0.03M-dibasic ammonium phosphate buffer/acetonitrile; 77:23 (2 ml/min) | 220nm | Formulations | Singh et al., 2000 |
| Waters C-18 3.9*300mm column | 0.1% TFA in $CH_3CN$ and 0.1% TFA in 99% $H_2O$/1%$CH_3CN$ (1.0ml/min) | 250nm | Formulations | - |
| AM-302 octadecylsilyl (15 cm × 4.6 mm) | aq. 10 to 40% acetonitrile containing 0.1% of trifluoroacetic acid 1 ml min-1. | 220nm | Serum/urine | Ueno et al., 1991 |
| ODS (15 cm × 4.0 to 4.6 mm) of (5 µm) - at and detection at | mobile phase of 87mM-NH4H2PO4 buffer (pH 6.5)-acetonitrile (77:23) 2 ml min-1 | 220 nm. | Injectables | Sutherland and Menon, 1987 |

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## 2.6 DNA

### 2.6.1 INTRODUCTION

Deoxyribonucleic acid (DNA) is a key constituent of the nucleus of living cells and is composed of building blocks called nucleotides consisting of deoxyribose sugar, a phosphate group and four nitrogenous bases-adenine, thymine, guanine and cytosine (Stryer, 1995). X-ray crystallography shows that a DNA molecule is shaped like a double helix, very much like a twisted ladder (Watson and Crick, 1953). The ability of DNA to contain and transmit genetic information makes it a very important biopolymer that has been the subject of intense physical studies in recent years. The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260nm, which is distinct from the peak exhibited by proteins at 280nm. A 50 μg/ml double stranded DNA sample on average gives an optical density at 260nm of 1.0. Fluorometry is widely regarded as one of the most sensitive molecular techniques for measuring DNA concentrations (Singer et al., 1997). The determination of DNA concentration using as little as possible is now important in vision research because of the need to analyse small samples of ocular tissue. Quantitative analysis of low concentrations of double stranded DNA is now feasible using fluorometry with newer fluorophores (fluorescent stains). Ethidium bromide preferentially binds to double stranded DNA by intercalation.

### 2.6.2 CALF THYMUS DNA

Calf thymus DNA was selected as a model DNA, can often serve as a reference for most plant and animal DNA because it is double stranded, is approximately 58% AT (42% GC). We have carried out our experiments using the highly purified sodium salt of calf thymus DNA, which was a gift from Biotechnology Department, M.S.University, in a lyophilised form with an average molecular weight of 60,000.

### 2.6.3 GENE DELIVERY

Genetic drugs are a class of therapeutic agents with considerable potential for the treatment of human diseases such as cancer and genetic disorders. Although numerous methods exist for effective in *vitro* gene delivery, current systems have limited utility for systemic applications. Viral systems, for

example, are rapidly cleared from the circulation, limiting transfection to first-pass"organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise transfection resulting from subsequent injections. In the case of nonviral systems such as plasmid DNA-cationic lipid complexes (lipoplexes), the large size and positively charged character of these aggregates also result in rapid clearance, and the highest expression levels are again observed in first-pass organs, particularly the lungs. The need for a gene delivery system for treatment of systemic disease is obvious. For example, for cancer gene therapy there is a vital need to access metastatic disease sites as well as primary tumors. Similar considerations apply to other systemic disorders, such as inflammatory diseases.

The design features for lipid based delivery systems that preferentially access such disease sites are increasingly clear. It is now generally recognized that preferential delivery of anticancer drugs to tumor sites following intravenous injection can be achieved by encapsulation of these drugs in large unilamellar vesicles (LUVs) exhibiting a small size (<100 nm diameter) and extended circulation lifetimes (circulation half-life in mice >5 h). The accumulation of these drug delivery systems at disease sites, including sites of infection and inflammation as well as tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue. The therapeutic index of the conventional or gene based (DNA or RNA transcripts) drug will be enhanced by delivering more biologically active drug to target cells / tissues and less to nontarget cells / tissues, to avoid drug-related toxicities. With gene-based drugs, however, delivery into appropriate cells represents only part of the problem; a number of intracellular barriers exist that can inhibit the biologic activity of gene-based drugs (Zabner et al., 1995; Dowty et al., 1995). It is not clear what role, if any, liposomes will play in overcoming these intracellular barriers. The potential of liposomes to systemically deliver DNA was recognized as early as the late 1970s, extensively reviewed in (Hug et al., 1991); however, gene-based drugs have presented interesting challenges for systemic delivery systems. First, gene-based drugs are highly susceptible to degradation by the nucleases present in plasma. Although liposomes have

the potential to encapsulate gene-based drugs and prevent inactivation by nucleases, procedures to efficiently encapsulate plasmid DNA in well defined, small liposomes or lipidic DNA particles have only recently been realized. Second, the efficacy of gene-based drugs is completely dependent on gaining entry into the target cell cytosol in an intact form. Therefore, for liposomes to be effective, they must incorporate agents that promote intracellular delivery. Third, for certain gene therapy approaches, such as those involving the delivery of suicide genes, systemic gene delivery systems must have the potential to selectively deliver gene-based drugs to specific target cells.

### 2.6.4 PRODUCTION OF LIPOSOMES FOR GENE DELIVERY

It is envisioned that the ideal liposomes for systemic gene delivery will encapsulate the DNA with high efficiencies, will protect the DNA from degradation by plasma nucleases, will have a narrow size distribution, averaging 100 nm or less in diameter, in order that the liposomes can access extravascular regions, and will have the potential to incorporate a wide range of lipids, especially lipids that promote fusion with cellular membranes and/or enhance liposome stability in the circulation.

The feasibility of passively encapsulating DNA in liposomes was demonstrated in the late 1970s. For example, high molecular weight DNA is entrapped in egg phosphatidylcholine liposomes by hydrating the lipid film in the presence of DNA (Hoffman et al., 1978). In a similar manner, metaphase chromosomes are passively entrapped in, or tightly associated with, egg phosphatidylcholineeholesterol (7:2, mol/mol) liposomes (Mukherjee et al., 1978). Alternatively, DNA can be encapsulated in cochleate lipid cylinders that are formed from the calcium-induced fusion of phosphatidylserine liposomes (Mannino et al., 1979). Reversed-phase evaporation procedures have also been employed to encapsulate plasmid DNAs with good but variable encapsulation efficiencies (Schaefer-Ridder et al., 1982, Nicolau et al., 1983). More recently, freeze drying methods have yielded DNA-containing multilamellar vesicles with encapsulation efficiencies of 5060% (Baru et al., 1995). Extrusion of the DNA containing multilamellar vesicles to reduce the particle size has resulted in poor recoveries of DNA containing liposomes. In the late 1980s, it was shown

that cationic lipids, when incorporated in dioleoylphosphatidyl ethanolamine (DOPE)-containing liposomes, could enhance the efficiency of gene delivery to cultured cells in vitro.

A gene delivery system containing an encapsulated DNA for systemic applications should therefore be small (<100 nm diameter) and must exhibit extended circulation lifetimes to achieve enhanced delivery to disease sites. This requires highly stable, serum-resistant DNA-containing liposome that does not interact with cells and other components of the vascular compartment. In order to maximize transfection after arrival at a disease site, however, the liposome should readily interact with cells at the site and should have the ability to destabilize cell membranes, to promote intracellular delivery of the plasmid. The behavior of liposomes can be modified by employing poly (ethyleneglycol) (PEG) coatings or by including other lipids such as cholesterol. In recent years, a large number of liposomal systems have been designed for the systemic delivery of conventional drugs (including chemotherapeutic agents and antibiotics). It is obviously desirable to develop liposomal systems for the systemic delivery of genetic drugs. This requires encapsulation of DNA in small lipid vesicle systems. Previous studies had shown that a PEG-lipid coating could prevent aggregation and fusion of LUVs induced by covalent coupling of protein to the vesicle surface. The properties of small size, serum stability, and low levels of cationic lipid and the presence of the PEG coating suggest that liposomes should exhibit extended circulation lifetimes and disease site targeting properties following intravenous administration.

The ability of a systemically administered gene therapy vector to exhibit extended circulation lifetimes, accumulate at a distal tumor site, and enable transgene expression is unique to liposomes. The flexibility and low toxicity of liposomes as a platform technology for systemic gene therapy allows for further optimization of tumor transfection properties following systemic administration. DNA has been encapsulated by a variety of methods, including reverse phase evaporation, ether injection, detergent dialysis in the absence of PEG stabilization, lipid hydration and dehydration-rehydration techniques and sonication.

Liposomal gene delivery has several benefits. It is less toxic and less immunogenic, activating low levels of complement than viral vectors and can transfer larger DNA molecules. They are easily produceable and can be administered repeatedly and efficiently and safely. However, their use is restricted by several factors where viral vectors would be more suitable. Primarily, the uptake of DNA into the cell nucleus is not as high as with viral vectors making them considerably less efficient. Many forms of the liposome-DNA complex can arise due to the rearrangement process giving variable effectiveness of DNA uptake and variable results from trial to trial.

### 2.6.5 LIPOSOMES FOR PROLONGED CIRCULATION

The use of liposomes for systemic drug delivery requires that the liposomes have the ability to avoid immediate uptake by phagocytic cells of the reticuloendothelial system (RES) and remain in circulation for extended periods of time in order to enhance the opportunity for the liposomal drugs to reach non-RES target tissues. A significant advance in the development of liposomal drugs has come with the use of specialized lipids, such as monosialoganglioside GM1or poly(ethylene glycol) (PEG)-modified phosphatidylethanolamine, that prolong circulation lifetimes when incorporated into liposomes (Klibanov et al., 1990; Blume and Cevc, 1990). It has been proposed that these PEG-lipid conjugates provide a 'steric stabilization' of the surface by virtue of the hydrophilic brush coat provided by the PEG polymer (Needham et al., 1992). This coat has been shown to inhibit serum protein binding to the liposomal surface (Semple et al., 1996), which would otherwise promote uptake by the RES, complement activation and destabilization of the liposomal membranes. It has been demonstrated that increased circulation lifetimes enhance the opportunity for liposomes, administered, systemically, to leave the vascular compartment and enter certain extravascular regions (Bakker-Woundenberg et al., 1992; Wu et al., 1993).

The ability to generate sustained circulating liposomal gene delivery systems using the PEG –lipid technology should prove useful for systemic gene delivery applications. For instance, the ability of long circulating liposomes to accumulate within tumors will be advantageous for cancer gene therapy applications involving tumor suppressor genes or suicide

genes. Furthermore, the avoidance of RES uptake, especially by Kupffer cells, the resident macrophages of the liver, would enhance the opportunity for liposomes to deliver genes to hepatocytes, the target cell of several gene therapies for blood protein deficiencies. The biodistribution of intravenously administered cationic liposome–plasmid DNA complexes is not appropriate for such systemic applications. For instance, it has recently been demonstrated that cationic liposome–plasmid DNA complexes, exhibiting strong positive zeta potentials, are cleared rapidly from the circulation (Mahato et al., 1995). These intravenously administered cationic liposome–plasmid DNA complexes [*N*-(2,3-bis(oleyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride or dimethyldioctadecylammonium bromide and DOPE-containing liposomes] are rapidly eliminated from the plasma, with 50–60% of the dose taken up by the liver within 5 min, and 20–30% of the dose taken up by the lung within 1 min, falling to 10% after 5 min. The cationic liposome–plasmid DNA complexes are predominantly taken up by the Kupffer cells in the liver. Moreover, a recent study has shown that cationic lipid–DNA complexes, harboring excess positive surface charge, are potent activators of the complement system, potentially a barrier to the efficient delivery of genes when using high lipid doses (Plank et al., 1996). Although there have been a few reports demonstrating the feasibility of using these complexes to deliver genes to a number of different tissues (such as the liver, lung, spleen, heart, skeletal muscle, kidney, uterus, bone marrow cells, peripheral blood and ovary) after intravenous administration (Osaka et al., 1996), the observed levels of gene delivery are low and often are not reproducible. This may be a consequence of the rapid elimination of the majority of the injected dose of cationic liposome–plasmid DNA complexes by the RES. Amphipathic PEG–lipid conjugates can stabilize DOPE-containing liposomes by inhibiting the fusogenic activity of these liposomes (Zhou et al., 1994).

In general, liposomes are effective delivery systems because they alter the pharmacokinetics of the free drug, leading to enhanced drug bioavailability to specific target cells that reside in the circulation or, more importantly, to extravascular disease regions. The ability to selectively deliver drugs to specific cells, such as tumor cells, within these regions will further enhance

the therapeutic index of liposomal drugs. Targeted delivery and improved therapeutic activity of liposomal drugs *in vivo* has been achieved by coupling site-directive targeting ligands, such as monoclonal antibodies, to the surface of liposomes by either covalent or non-covalent methods. A significant advance in this area has been the advent of novel PEG–phosphatidylethanol- amine lipids that allow targeting ligands to be conjugated at the distal ends of the PEG spacer. These conjugates increase target cell binding in vitro, as well as prolong circulation times. For liposomal gene delivery systems, targeting ligands need to function not only to increase the binding of the liposomes to specific target cells, such as hepatocytes, but also to promote the cellular uptake of the liposomes via an endocytic pathway. Although these are early stages for liposomal gene delivery systems, several of the advances made in liposomal drug delivery technologies can be directly applied to these systems. Noteworthy is the use of exchangeable PEG-lipid conjugates to stabilize the plasmid DNA-containing lipid-based carriers in the circulation. This should expedite the development of systemic liposomal gene delivery systems that exhibit targeted and enhanced intracellular delivery.

The aim of gene therapy is to replace genes in the genome with altered ones with desired characteristics that will be expressed by the target cell. This has two main applications. Firstly, in the treatment of congenital and occasionally acquired diseases whereby the body produces faulty proteins by expression of incorrect or faulty genes causing disturbance to cellular processes. This results in cell malfunction or in some cases alteration of the replication cycle causing uncontrollable cell division as in certain cancers. Secondly, gene therapy has potential in preventing the rejection of transplanted tissue, either by expressing genes to prevent rejection or by suppressing genes, which cause rejection.

Liposome-DNA complex uptake by two possible cell uptake mechanisms of the liposome-DNA complex. Either by endocytosis followed by destruction of the endosome within the cell. Alternatively by direct fusion with the cell plasma membrane. It has been shown that the first method is that most frequently occuring with only 2% uptake by direct fusion.

1. Entrance of DNA into cell nucleus: Important step determining efficiency of delivery process.
2. DNA expression by cell

Liposome-DNA complexes by themselves are not targetted to specific cell types. The incorporation of certain molecules into the lipoplex surface, however, can target the molecule to certain cells. For example, the ancoring of carbohydrates to the lipoplex surface can target it to tumour cells because transformed; tumour cells possess greater amounts of lectins on their surface, which bind carbohydrates.

### 2.6.6 QUANTITATIVE METHODS

**2.6.6.1 Ultraviolet (UV) spectrophotometry** can be used to determine the quantity of DNA present in a sample and estimate its purity. It works because nucleic acids and proteins have different absorbance spectra. DNA and RNA both strongly absorb UV light with a maximum absorbance at 260 nm. This allows the quantification of these molecules by UV absorbance at concentrations as low as 2μg/ml. A solution of pure doublestranded DNA with a concentration of 50μg/ml has A260 of 1.0.

Measurement of the absorbance of a nucleic acid solution at wavelengths other than 260 nm is useful for characterizing purity. Proteins absorb UV light maximally at 280 nm, and the A260:A280 ratios of a DNA solution are often used as indicators of protein or RNA contamination. A260:A280 ratio from a preparation of pure double-stranded DNA should be between 1.8 and 1.9. Higher ratios are often due to RNA contamination; lower ratios can indicate the presence of protein. This method of quantitation is accurate only if the concentration of DNA is greater than 500ng/ml.

**2.6.6.2 Chemiluminescence method:** (Chen et al., 2003; Pirami et al., 1994).

A highly sensitive flow-injection chemiluminescence method for the determination of calf thymus DNA and herring sperm DNA was developed. The method is based on the chemiluminescence reaction of Rhodamine B-cerium(IV)-thermally denatured DNA in sulfuric acid medium. The procedure allowed quantification of DNA in the range 0.026-260 ng/ml for calf thymus DNA and 0.05-50 pg/ml for herring sperm DNA with correlation

coefficients of 0.9998 and 0.9996 (both $n$ = 11), respectively. The detection limits were 6.5 pg/ml for calf thymus DNA and 0.043 pg/ml for herring spermDNA.

### 2.6.6.3 Fluorescence method

Fluorometers are especially designed to measure low concentrations of DNA, even in the presence of RNA. Dyes that bind specifically to dsDNA (double stranded DNA) extend the ability to determine concentrations of DNA too low to be analysed by $A_{260}$. Reliable quantification of DNA is an essential issue for researchers in biochemistry and molecular biology. Among the quantitative methods the assay of the intensity ultraviolet (UV)-induced fluorescence emitted by the intercalating dye, ethidium bromide (EtBr) is commonly applied. The dye is unaffected by the differences in the base composition and as little as 1 to 5 ng of DNA can be estimated by this method [Ausubel et al., 1987].

This method relies on the selective binding of Hoechst 33258 dye (bisbenzamide) to the minor groove of DNA. The binding of the dye to DNA shifts the fluorescence emission of the dye and allows the accurate measure of DNA concentrations. In the absence of DNA, the excitation spectrum of H33258 peaks at 356 nm and the emission spectrum peaks weakly at 492-nm. When H33258 binds to DNA, these peaks shift to 365-nm excitation and 458-nm emission. In the cuvette well, the sample is exposed to filtered light (365 ± 7nm) from a mercury lamp. This light excites the DNA-dye complex, causing light that peaks at 458 nm to be emitted. An emission filter in front of the photodetector allows only fluorescence at 460 nm ± 15 nm to register. Thus the measured fluorescence is an indicator of the DNA concentration.

Several methods have been used to analyse the structural properties of nucleic acids. These include UV absorption, circular dichroism, vibrational (Raman and infrared), nuclear magnetic resonance, X-ray and fluorescence spectroscopy. While NMR and X-ray diffraction spectrometry provide the highest structural detail into the three-dimensional fold of a biomolecule, their applications to DNA are severely curtailed by the enormous size and flexibility of the analyte, resulting in poor sensitivity and resolution.

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